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# **NEUTROPHIL FUNCTION IN CHRONIC PERIODONTITIS**

by

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## Abstract

Neutrophil function in the pathogenesis of chronic periodontitis was investigated. A case-controlled longitudinal intervention study of patients with chronic periodontitis and matched healthy controls was performed. Peripheral blood neutrophils from patients released more IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  in response to periodontally-relevant bacteria than controls. Hyper-reactive *Fusobacterium nucleatum*-stimulated neutrophil IL-8, IL-6 and TNF- $\alpha$  release from patient cells normalised to control levels following successful therapy. Hyper-reactive Fc $\gamma$ R-stimulated IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  and *Porphyromonas gingivalis*-stimulated IL-1 $\beta$  release by patient cells persisted after therapy. Patient neutrophils displayed hyper-active and hyper-reactive superoxide release that normalised to healthy control levels post-therapy. Although neutrophil extracellular trap release was unchanged in periodontitis, patient neutrophils demonstrated impaired directional chemotactic accuracy, speed and velocity. Studies on control neutrophils demonstrated that physiologically-relevant concentrations of C-reactive protein (CRP) inhibited baseline reactive oxygen species (ROS) generation, and reduced Fc $\gamma$ R-stimulated superoxide and *F. nucleatum*-stimulated luminol/isoluminol detectable ROS. However, CRP enhanced *F. nucleatum*-stimulated neutrophil superoxide release. The data demonstrate that chronic periodontitis is characterised by dysregulated neutrophil function, notably heightened cytokine and superoxide production and impaired chemotaxis. Furthermore, mildly elevated CRP levels in periodontitis could play a role in modifying the neutrophil respiratory burst and provide a link with periodontitis-associated systemic disease.

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# Abbreviations

AA	Amino acid
AAP	American Academy of Periodontology
ACPA	Anti-citrullinated protein antibody
AFU	Arbitrary fluorescence units
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Analytical ultracentrifugation
BBC	Birmingham and the Black Country
BCHCT	Birmingham Community Healthcare Trust
BOP	Bleeding on probing
BPE	Basic Periodontal Examination
BSA	Bovine serum albumin
C1q	C1q complex
C3b	Complement components C3b
C5a	Complement component C5a
CAL	Clinical attachment loss
CD	Cluster of differentiation
CGD	Chronic granulomatous disease
CID	Collision-induced dissociation
CINC-1	Cytokine-induced neutrophil chemoattractant 1
ConA	Concanavalin A
COPD	Chronic obstructive pulmonary disease
CRN	Comprehensive local research network
CRP	C-reactive protein
CVD	Cardiovascular disease
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Extinction coefficient
EFP	European Federation of Periodontology
ELISA	Enzyme-linked immunosorbent assay
Fc $\gamma$ R	Fc-gamma receptor
fMLP	N-formyl-Met-Leu-Phe
FPR	Formylpeptide receptor
g	Gram
GCF	Gingival crevicular fluid
GI	Gingival index
GM-CSF	Granulocyte/macrophage colony stimulating factor
GPBS	Glucose-supplemented PBS
GRO- $\alpha$	Growth-related oncogene- $\alpha$
GSH	Glutathione
GSSG	Glutathione disulphide



H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HAP	Hydroxyapatite
HbA1C	Glycated haemoglobin
HGF	Hepatocyte growth factor
HNK	Hemin-N-acetylmuramic acid-vitamin K
HOCl	Hypochlorous acid
hr	Hours
HRP	Horseradish peroxidase
hsCRP	High-sensitivity C-reactive protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP-10	Interferon- $\gamma$ -inducible protein-10
IQR	Interquartile range
IRAS	Integrated Research Application System
I-TAC	IFN-inducible T cell chemoattractant
l	Litre
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTB <sub>4</sub>	Leukotriene B <sub>4</sub>
M	Molar
Max	Maximum
MCP-1	Monocyte chemoattractant protein-1
mCRP	Modified C-reactive protein
MIG	Monokine induced IFN $\gamma$
min	Minute
MIN	Minimum
MIP-1	Macrophage inflammatory protein 1
mm	Milimetre
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MW	Molecular weight
MWCO	Molecular weight cut-off
N	Newton
NADPH	Nicotinamide adenine dinucleotide phosphate
NAM	N-acetylmuramic acid
NaN <sub>3</sub>	Sodium azide
NET	Neutrophil extracellular trap
NHS	National Health Service
NIHR	National Institute of Health Research
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NS	Not significant
OD <sub>600nm</sub>	Optical density at 600 <sub>nm</sub>
$\cdot$ OH	Hydroxyl radical
O <sub>2</sub> <sup>-</sup>	Superoxide anion
OSM	Oncostatin M
PAD	Peptidylarginine deiminase

PBS	Phosphate buffered saline
PDK1	Phosphodinositide-dependent kinase
PG	Prostaglandin
PI	Plaque index
PI <sub>3</sub> K	Phosphoinositide 3-kinase
PIP <sub>3</sub>	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PPAD	<i>P. gingivalis</i> peptidylarginine deiminase
PPD	Probing pocket depth
Q1	First quartile
Q3	Third quartile
R&D	Research and development
rcf	Relative centrifugal force
REC	Research ethics committee
RLU	Relative light units
RM&G	Research Management & Governance
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RSD	Root surface debridement
s	Seconds
SD	Standard deviation
SOD	Superoxide dismutase
TBS	Tris-buffered saline
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
UK	United Kingdom
Y-IgG	IgG-opsonised yeast

# **CHAPTER 1**

## **INTRODUCTION**

## **1.1 PERIODONTAL DISEASES**

The periodontal tissues form the supporting structures of the human dentition and comprise of alveolar bone, root cementum, periodontal ligament, and gingival connective tissues covered by crevicular and junctional epithelium. The crevicular epithelium is highly porous with gingival crevicular fluid (GCF) continually released into the gingival crevice as a serum transudate arising from the surrounding vasculature. In response to intra-oral microbial exposure the periodontal tissues are readily infiltrated by polymorphonuclear leukocytes (neutrophils; section 1.5) recruited from the periodontal vessels into the periodontal tissues. Neutrophils are also present within GCF which becomes a serum exudate during gingival and periodontal inflammation.

### **1.1.1 Chronic periodontitis**

Chronic periodontitis is a common inflammatory condition affecting the periodontal tissues resulting in irreversible apical migration of the junctional epithelium, loss of periodontal attachment and ultimately tooth loss (Löe *et al.* 1986). Chronic periodontitis represents a pathological progression from gingivitis which, in contrast, is a reversible inflammation in response to intra-oral plaque bacteria that is restricted entirely to the gingival soft tissues without loss of periodontal connective tissue attachment. Chronic periodontitis is clinically and pathologically distinct from other less common periodontal diseases such as aggressive and necrotizing ulcerative periodontitis (Milward & Chapple 2003).

The UK Adult Dental Health Survey has highlighted that approximately half of all dentate adults have evidence of current periodontal disease, with approximately 66% of adults aged  $\geq 55$  yrs having evidence of loss of periodontal attachment (White *et al.* 2012). Periodontal disease is the leading cause of tooth loss in the world (Papapanou 1999), with chronic periodontitis the most common form representing the vast majority of all periodontal diseases (Demmer & Papapanou 2010). It is important to appreciate that chronic periodontitis is a chronic, largely asymptomatic condition that may go unnoticed by the patient unless regularly screened by a dental professional, and this is likely to be reflected in the high worldwide prevalence of this disease and subsequent impact on healthcare costs.

### **1.1.2 Clinical diagnosis of chronic periodontitis**

General Dental Practitioners screen for the presence of periodontal diseases by performing a Basic Periodontal Examination (BPE) followed, where appropriate, by a detailed periodontal examination. The latter includes measuring periodontal probing pocket depths and recession (together measuring clinical attachment loss), tooth mobility, occlusal assessment, the presence of bleeding on probing and plaque scoring along with appropriate radiographic examination. Once identified, periodontal diseases are then classified in order to reflect the differing pathogeneses of these conditions and also to provide an indication as to the most appropriate treatment strategy (Armitage 1999). Chronic periodontitis may be defined as the presence of at least two non-adjacent sites per quadrant with probing pocket depths  $\geq 4\text{mm}$  along with radiographic bone loss  $\geq 30\%$  of the root length (non-first molar or incisor sites) in accordance with the consensus criteria of the European Federation of Periodontology (Tonetti & Claffey 2005). Periodontal health is regarded as being consistent with probing pocket depths  $< 4\text{mm}$  (Matulienė *et al.* 2008), bleeding scores  $< 10\%$  (Lang *et al.* 1986, 1990) and plaque scores  $< 20\%$  (Lang & Tonetti 1996). The presence of gingival recession without pocketing represents historical attachment loss that may no longer be active, or tissue loss due to traumatic toothbrushing.

## **1.2 THE PATHOGENESIS OF CHRONIC PERIODONTITIS**

The presence of plaque bacteria is a pre-requisite for gingivitis (Löe *et al.* 1965, Theilade *et al.* 1966) however gingivitis does not always progress to chronic periodontitis. Approximately 10% of the population are resistant to the development of periodontitis (Löe *et al.* 1986). The presence of pathogenic plaque bacteria within a susceptible host results in a pathological transition from gingivitis to chronic periodontitis, the pathogenesis of which is currently poorly understood, but is likely to be caused either directly by periodontal pathogens or alternatively as a result of a dysregulated host inflammatory-immune response (Van Dyke 2008).

### **1.2.1 The role of bacteria**

Whilst intra-oral bacteria are present naturally, plaque deposits accumulate around the teeth in the absence of adequate oral hygiene measures. Supra-gingival plaque is a risk factor for dental caries (Mannaa *et al.* 2013) but also eventually leads to the accumulation of a plaque

biofilm sub-gingivally (Kornman 1986). Subgingival plaque bacteria form a biofilm within the gingival crevice through the interaction of early and eventually late bacterial colonisers, and the salivary molecules of the acquired pellicle surrounding the root dentine (Walket & Sedlacek 2007). The subgingival microbial flora is complex and it has been estimated that approximately 1,200 species of bacteria have the potential to colonise the periodontium at any one time (Paster *et al.* 2001). However only relatively few periodontal bacteria are directly involved in periodontal pathogenesis, and a single causative pathogen has not been identified (Socransky *et al.* 1984). The healthy subgingival microflora consists mainly of facultative (both aerobic and anaerobic capabilities) anaerobic Gram positive bacteria, with only approximately 5% spirochaetes and motile rods. As the transition from periodontal health to gingivitis and chronic periodontitis progresses, a microbial shift occurs with a greater proportion of anaerobic, Gram negative spirochaetes and other motile bacteria (Listgarten *et al.* 1978, Marsh 1994). In chronic periodontitis approximately 75% of bacteria are Gram negative, of which 90% are strict anaerobes (Marsh 1994).

There are three main theories describing the role of bacteria in the pathogenesis of periodontitis. The theory that a limited number of specific bacterial species trigger periodontal inflammation is known as the “specific plaque hypothesis” (Loesche 1976). Bacteria have the ability to cause disease by directly releasing damaging proteolytic and hydrolytic enzymes that may also directly damage the surrounding tissues. Alternatively bacteria may also induce a dysregulation of the host response as a result of interaction with surrounding host cells (Dale 2002, Milward *et al.* 2007). Gram-negative anaerobic bacterial pathogens such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are widely regarded as key aetiological species (Socransky *et al.* 1998) and are considered important regulators of the host response. This is due to their ability to invade cells and tissues therefore promoting bacterial survival, modulating virulence properties along with dysregulation of the inflammatory response of the invaded cell (Han *et al.* 2000, Eick *et al.* 2006). In addition, *F. nucleatum* coaggregates with other periodontal bacteria via coaggregation receptors, thereby acting as a bridge between early and late colonisers (Rickard *et al.* 2003). In addition, anaerobic secondary colonisers, such as *P. gingivalis*, are unable to survive in the planktonic state unless coaggregated to *F. nucleatum* (Diaz *et al.* 2002) therefore highlighting the importance of *F. nucleatum* not only with regards to the development of dental plaque, but

also in mediating the microbial shift from a non-pathogenic to a pathogenic microflora (Kolenbrander *et al.* 1995; section 1.2.1). *Porphyromonas gingivalis* is strongly associated with periodontal disease pathogenesis (Socransky *et al.* 1998) and has many different pro-inflammatory activities. These include the secretion of destructive proteinases (O'Brien-Simpson *et al.* 2003), along with effects on cytokine induction, neutrophil chemotaxis and contributing to alveolar bone loss by stimulating bone resorption, inducing bone destruction and inhibiting bone formation (Lamont & Jenkinson 1998).

The second theory, the “non-specific plaque hypothesis,” proposes that plaque bacteria will become pathogenic when a threshold volume is reached, irrespective of its constitution (Theilade 1986). This occurs by effectively disrupting host-bacterial homeostasis and triggering a host-inflammatory response through non-specific mechanisms such as activation by bacterial lipopolysaccharide (LPS; Marsh 2005). Indeed, there is reproducible evidence using a 21-day gingivitis model describing the relationship between plaque accumulation and gingivitis showing that plaque mass alone does induce gingival inflammation (Löe 1973). Whilst the “non-specific plaque hypothesis” is important in the development of gingivitis, it is likely that specific bacteria play a larger role in periodontal pathogenesis. This is because, in contrast to gingivitis, periodontal disease severity rarely directly correlates clinically with the amount of plaque present, along with different thresholds of plaque control required for different patients with regards to ultimately achieving periodontal stability. As previously discussed, a number of key periodontal pathogens are considered to be important in the pathogenesis of chronic periodontitis including *P. gingivalis* and *F. nucleatum* (Socransky *et al.* 1998). This is reflected in their widespread selection in relation to published literature and current studies investigating the host response in chronic periodontitis. However, as previously stated, periodontal pathogens form bacterial complexes *in-vivo* rather than existing in isolation, and therefore a third hypothesis was postulated by Haffajee in 1991, namely the “environmental plaque hypothesis” which suggests that the entire subgingival microbial environment is involved in periodontal pathogenesis (Haffajee *et al.* 1991).

### **1.2.2 The role of the host response**

The two main components of the host response to plaque bacteria are the innate and the acquired immune responses. The innate immune system is constantly in function and occurs

rapidly, whereas additional time is required for generation of the acquired immune response. Both the innate and acquired immune responses have the potential to cause collateral tissue damage if not regulated or controlled correctly. Early gingival inflammation largely involves the innate system whereas the acquired system also becomes involved in moderate-to-advanced gingivitis and periodontitis i.e. longer-term, chronic disease (Teng 2003).

The pathogenesis of chronic periodontitis, including the transition from gingivitis to periodontitis, involves an imbalanced host-derived inflammatory-immune response, coordinated by locally and systemically-derived cytokines (Page 1991, Preshaw *et al.* 2011, Deo & Bhongade 2010). Crevicular epithelial cells and tissue-resident macrophages within the periodontal connective tissues are involved in the recognition of plaque bacteria via Toll-like receptors (TLR) which bind bacterial components such as lipopolysaccharide (LPS), lipotechoic acids and/or bacterial DNA. Receptor binding then activates intra-cellular signalling pathways (e.g. via transcription factors such as NF- $\kappa$ B) resulting in the secretion of a number of key cell signalling molecules into the surrounding tissues, including chemokines (e.g. IL-8) and cytokines (e.g. IL-6, IL-1 $\beta$ , TNF- $\alpha$ ) that are involved in the recruitment and activation of immune cells. Secreted cytokines and chemokines subsequently diffuse from the site of production, creating a gradient of concentrations and resulting in the recruitment and infiltration of inflammatory cells such as neutrophils from the surrounding vasculature. Neutrophils are the most abundant defence cell (section 1.5) and once recruited to the site of infection, kill bacteria by either intra- or extra-cellular methods. In addition, macrophages scavenge dead cells (both bacterial and host) and also play an important role in bridging the gap between innate and acquired immunity by acting as antigen presenting cells leading to the development of acquired immunity.

In periodontal health the junctional epithelium naturally forms an epithelial attachment via hemi-desmosomes to the tooth enamel derived from ectoderm embryologically (inner enamel epithelium). However once attachment loss occurs, either through periodontitis or recession, irreversible apical migration and elongation of the junctional epithelium occurs which then migrates onto root cementum, derived from mesenchyme (Pitaru *et al.* 1994). This situation is similar to that of a compound fracture of bone where direct communication exists between the external environment and an internalised part of the skeleton, namely the tooth with its



investing alveolar bone emerging through an epithelial lining. Migration of the junctional epithelium allows subgingival bacteria to be adjacent to the cementum and junctional epithelium that was previously associated with attached gingival connective tissue (Nanci & Bosshardt 2006). Therefore the junctional epithelium is permeable to GCF carrying host defence cells (mainly neutrophils) and various other components of the inflammatory-immune response such as complement and antibody. However the downside of this arrangement is that bacterial products can then also pass back into the gingival tissues and stimulate both local and systemic inflammatory reactions (section 1.3). Subgingival plaque accumulation leads to micro-ulceration of the crevicular epithelium accounting for the clinical sign of bleeding on probing, as well as further propagating inflammatory-immune responses within the surrounding connective tissues and systemic circulation.

Bacteria are essential to the pathogenesis of periodontitis however plaque bacteria alone are insufficient to cause periodontal disease. The presence of supragingival plaque deposits is inversely associated with the severity of periodontal attachment loss with a relative risk of 0.2 (Grossi *et al.* 1994), with the majority of periodontal tissue damage attributed to a dysregulated host response (Page *et al.* 1997). Whilst host responses are designed to be protective, the balance between bacterial-host interactions is delicate and under- or over-activity potentially results in tissue destruction. In health a balance is achieved between the oral bacteria and the host response, however such equilibrium may easily be disrupted leading to inflammation and tissue damage. Chronic periodontitis is predominantly characterised by a neutrophilic inflammatory response which can represent both the first line of defence in the innate immune response and part of the effector cell arm of the acquired immune response. A “hyper-inflammatory” neutrophil phenotype is considered central to the pathogenesis of chronic periodontitis with subgingival plaque resulting in an exaggerated inflammatory-immune response causing disease. Subsequent failure to remove plaque bacteria via adequate oral hygiene and periodontal treatment causes chronicity of the inflammatory lesion. Neutrophilic “hyper-inflammation” is also a characteristic of many systemic inflammatory diseases associated with chronic periodontitis such as diabetes and cardiovascular disease (section 1.3) and may represent mechanisms with which periodontitis contributes to co-morbidity.

### 1.3 RELATIONSHIP WITH SYSTEMIC DISEASE

As a result of periodontal inflammation due to plaque accumulation, the crevicular epithelium ulcerates (section 1.2.2) and exposes a surface area of exposed gingival connective tissue and associated vasculature similar in size to that of the palm of the hand (8-20cm<sup>2</sup>; Hujoel *et al.* 2001). As a result, periodontal bacteria and their virulence factors have the potential to enter the surrounding tissues and vasculature causing not only local but also systemic acute-phase and inflammatory-immune consequences (Loos 2005, Seymour *et al.* 2007).

The “focal infection theory” was first postulated by Hippocrates (460-370BC) who was first to recognise that localised sites of infection, such as the periodontal tissues, may disseminate to distant locations which may then result in secondary infections that initiate, sustain or worsen systemic disease (Graham 1931, Pallasch & Wahl 2000). Hippocrates was subsequently said to have reported the successful treatment of rheumatoid arthritis by extraction of an infected tooth. In addition, Elie Metchnikoff (1845-1916), who discovered neutrophil phagocytes, also drew attention to bacteria as potential causes of both septic and aseptic inflammatory disease (Metchnikoff 1907). A British surgeon William Hunter (1861-1937) subsequently identified oral sepsis as a cause of systemic diseases (Hunter 1900, 1901) and later claimed that “*the worst cases of anaemia, gastritis, colitis, obscure fevers, nervous disturbances of all kinds from mental depression to actual lesions of the cord, chronic rheumatic infections, kidney diseases are those which owe their origin to or are gravely complicated by the oral sepsis produced by these gold traps of sepsis*”, referring to the presence of dental restorations (Hunter 1923). Therefore, it has long been recognised that periodontal diseases have the potential to influence the systemic health of patients. More recent evidence suggests that chronic periodontitis serves as a source of pro-inflammatory mediators resulting in an exaggerated systemic inflammatory-immune response which may then contribute to the development of systemic disease (Tonetti & Van Dyke 2013).

Chronic periodontitis is associated with many chronic inflammatory diseases including type-2 diabetes (Chapple & Genco 2013), atherogenic cardiovascular disease (Dietrich *et al.* 2013), rheumatoid arthritis (DePablo *et al.* 2009), chronic kidney disease (Sharma *et al.* 2014) and chronic obstructive pulmonary disease (Linden & Herzberg 2013). Evidence implicating chronic periodontitis in the causal pathway of chronic diseases varies according to the

condition, and the European Federation of Periodontology (EFP) and American Academy of Periodontology (AAP) recently held a joint workshop compiling a number of comprehensive systematic reviews (Tonetti & Kornman 2013). The strongest evidence implicating chronic periodontitis in the causal pathway of chronic diseases relate to a bi-directional relationship with type-2 diabetes in which patients with periodontitis are 6-times more likely to have poor glycaemic control which improves following periodontal treatment (Taylor *et al.* 2001). Periodontal examination and treatment is now included in the consensus report of the joint European Federation and American Academy of Periodontology regarding the joint dental and medical management of patients diagnosed with type-2 diabetes and typically resulting in an approximately 0.4% reduction in HbA1C at 3 months (Chapple & Genco 2013). Meta-analyses have also demonstrated that patients with chronic periodontitis have a 34% increased risk of developing cardiovascular disease (Blaizot *et al.* 2009) and are 2.9 times more likely to suffer from ischaemic stroke (Janket *et al.* 2003), with periodontal treatment resulting in consistent and progressive reductions in systemic inflammation and biomarkers of disease risk, including improvements in vascular endothelial function and flow-mediated dilation (Tonetti *et al.* 2007). The impact of chronic periodontitis on systemic health is further emphasised and associated with an overall  $\leq 85\%$  increased risk of premature death resulting from all-cause mortality (Garcia *et al.* 1998).

Systematic reviews and meta-analyses have demonstrated that patients with chronic periodontitis have raised levels of inflammatory mediators within the circulation including acute phase reactants such as C-reactive protein (CRP), that are lowered following periodontal treatment (Paraskevas *et al.* 2008). Systemic CRP concentrations are widely used medically to assess the presence of systemic inflammation and also to determine cardiovascular disease risk (Ridker 2003; section 1.7.1). Undiagnosed or untreated periodontal disease is therefore likely to increase the systemic inflammatory burden and hence the risk of co-susceptibility and co-morbidity. Similarly, both periodontitis and other chronic inflammatory diseases are associated with a “hyper-inflammatory” neutrophil phenotype (section 1.2.2) and this is likely to further contribute to co-susceptibility, co-morbidity and possibly represent a causal link between periodontal and other prevalent systemic inflammatory-immune diseases. The consensus view currently is that it is likely that the association between periodontitis and systemic disease are linked as a result of a repeated bacteraemia during eating, toothbrushing

and daily function, triggering a systemic inflammatory-immune response, with persistent chronic entry of oral bacteria into the blood stream arising from the periodontium (Tonetti & Kornman 2013).

#### **1.4 RISK FACTORS FOR CHRONIC PERIODONTITIS**

Risk factors increase the probability of a disease developing in a given individual. Risk factors for chronic periodontitis may be classified as locally or systemically-derived (Van Dyke & Sheilesh 2005) and have the potential to alter the microflora and/or the host response. The host's inflammatory-immune response to bacteria is largely genetically determined, however environmental and lifestyle factors play important modifying roles (Tonetti & Chapple 2011).

The most important modifiable risk factor is oral hygiene (plaque control), since removal of the initiating factor avoids activating the chronic non-resolving inflammation that characterizes chronic periodontitis and gingivitis. Local risk factors for periodontitis relate directly to plaque accumulation or barriers to biofilm removal, for example the presence of calculus, furcation involvement and overhanging or deficient restoration margins. There are also many systemic risk factors which impact upon the systemic inflammatory-immune response and therefore the risk of chronic periodontitis, and also vice-versa in a bi-directional manner (section 1.3; Taylor *et al.* 2001).

Natural history studies observing the progression of periodontitis including longitudinal studies of tea plantation workers in Sri Lanka have demonstrated three subsets of the population relative to the development of periodontitis, namely high risk (~10%) and resistant (~10%) groups, with the majority (~80%) classified retrospectively as having medium risk of developing periodontitis (Löe *et al.* 1978, 1986). Genetic susceptibility is regarded as representing approximately half of chronic periodontitis disease risk and prevalence based on twin studies (Michalowicz 1994), with several single gene polymorphisms subsequently investigated and the recent use of genome-wide association studies (Vaithilingam *et al.* 2014).

In contrast to genetic factors, many local and systemic risk factors are modifiable and therefore have the potential to minimize disease risk. The most important modifiable risk

factor is a patient's oral hygiene, with full-mouth plaque scores <20% being associated with continued periodontal stability (Lang & Tonetti 1996). In addition, smoking is considered a significant risk factor for chronic periodontitis (Palmer *et al.* 2005) with smokers 2-3 times more likely to develop periodontitis (Dietrich *et al.* 2007), and also exhibiting poorer responses to treatment (Heasman *et al.* 2006). Whilst cigarette smoke extract generally decreases neutrophil reactive oxygen species (ROS) generation, at higher concentrations possibly seen in heavy smokers it also stimulates peripheral blood neutrophil extracellular ROS release in a dose-dependent manner (Matthews *et al.* 2011) which may contribute to a "hyper-inflammatory" neutrophil phenotype. Similarly, type-2 diabetes displays a unique bi-directional relationship with chronic periodontitis (Taylor 2001) and is similarly associated with enhanced peripheral blood neutrophil reactive oxygen species (ROS) (Wong *et al.* 2002), cytokine release (Hatanaka *et al.* 2006), and oxidative stress (Allen *et al.* 2011). The presence of a "hyper-inflammatory" neutrophil phenotype within periodontitis (section 1.3) may result in increased neutrophil ROS release leading to oxidative stress (Sies 1986, Halliwell 1990). Increased antioxidant intake may therefore help to restore this balance and therefore reduce periodontal (and other systemic) disease risk (Chapple 2009). Micronutritional approaches to reduce periodontal disease risk by modifying ROS activity have since been highlighted as novel therapeutic approaches (Van der Velden *et al.* 2011). Additional systemic risk factors include psychosocial stress that has the ability to not only modify the growth of periodontal bacteria (Roberts *et al.* 2002, 2005) but also impact upon neutrophil responses (Ellard *et al.* 2004, 2005). Given the importance of the neutrophil in the host response in periodontitis (section 1.2.2), qualitative and quantitative defects in neutrophil numbers (e.g. neutropenia) and function (e.g. Papillon-Lefèvre syndrome) increase the risk of very severe forms of periodontal disease, along with serious systemic illness, particularly in young children (Kanthimathinathan *et al.* 2013).

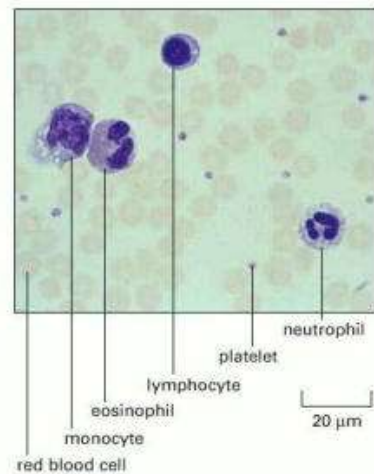
## 1.5 NEUTROPHILS IN HEALTH & DISEASE

Neutrophils are terminally differentiated leucocytes derived from myeloblasts and produced in the bone marrow (Amulic *et al.* 2012). They account for 50-60% of all leukocytes in peripheral blood, with normal mean numbers of  $\sim 4.4 \times 10^6$  cells/ml. Neutrophils were traditionally regarded as being relatively short-lived however more recent evidence from *in-vivo* life expectancy studies have demonstrated neutrophil half-lives as long as 5.4 days

(Pillay *et al.* 2010) and produced in large numbers ( $1-2 \times 10^{11}$  cells generated per day; Borregaard *et al.* 2010). They are approximately 9-10 $\mu\text{m}$  in diameter and have a characteristic multilobed nucleus (Figure 1.1). Neutrophils also possess unique staining characteristics with haematoxylin and eosin histological or cytological preparations and stain a neutral pink, which allows them to be distinguished from other types of polymorphonuclear leucocytes including basophils and eosinophils (Alberts *et al.* 2002; Figure 1.2).



**Figure 1.1. Transmission electron micrograph image of a human neutrophil showing the characteristic multilobed nucleus and granule-dense cytoplasm** (Brinkmann & Zychlinsky 2007).



**Figure 1.2. A neutrophil with characteristic multilobed nucleus is visible alongside other immune cells within a light photo micrograph of a blood smear with Romanowsky stain** (Alberts *et al.* 2002).

The first line of cellular inflammatory-immune defence involves the recruitment of large numbers of neutrophils from the systemic circulation to the site of tissue damage or infection. Neutrophils kill invading bacteria by phagocytosis, release of intra- and extracellular ROS (Chapple & Matthews 2007), neutrophil extracellular traps (NETs; Brinkmann *et al.* 2004;

section 1.5.4.5) as well as hydrolytic and proteolytic enzymes and antimicrobial peptides (Borregaard & Cowland 1997). Neutrophils also play an active role in coordinating the inflammatory-immune response through the release of cytokines including IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  (Cassatella *et al.* 1997, 1999).

### **1.5.1 Neutrophil cytokine release**

For a long time, monocytes and lymphocytes were primarily regarded as the predominant source of pro-inflammatory cytokines in inflammatory lesions. By contrast, neutrophils were initially thought to produce only IL-8 (Bazzoni *et al.* 1991, Cassatella *et al.* 1992, 1993, Arnold *et al.* 1994, Wei *et al.* 1994, Altstaedt *et al.* 1996), possibly as a result of containing relatively few ribosomes and little endoplasmic reticulum. However, neutrophils have since been shown to release physiologically-relevant concentrations of important cytokines including IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  (Cassatella *et al.* 1997, 1999, Kasama *et al.* 2005) and play an active role in co-ordinating the inflammatory-immune response, in addition to their traditional antimicrobial activity. It is now believed that cytokines within the periodontium interact together in a network thereby modulating the inflammatory-immune response and the pathogenesis of periodontitis (Preshaw & Taylor 2011). Neutrophils are known to release a large number of cytokines (Table 1.1) and chemokines (Table 1.2) in response to a variety of conditions, which have a variety of intercalated functions within a network in the pathogenesis of chronic periodontitis (Tables 1.1 and 1.2)

### **1.5.2 Neutrophil recruitment from the systemic circulation**

Epithelial cells of the crevicular epithelium along with resident tissue macrophages and neutrophils respond to the presence of bacteria by releasing cytokines and chemokines thereby activating the inflammatory-immune response. Cytokines and chemokines, along with bacterial products themselves such as fMLP (N-formyl-Met-Leu-Phe) and lipopolysaccharide (LPS) subsequently diffuse into the periodontal tissues activating endothelial cells within the surrounding vasculature (Chen *et al.* 2009). TNF- $\alpha$  and IL-1 $\beta$  subsequently upregulate endothelial cell adhesion molecules (intracellular adhesion molecules 1 & 2 and vascular cell adhesion molecules) that interact with neutrophil integrins (Kettritz *et al.* 2004, Zhang *et al.* 2011). Initial binding between the neutrophils and vascular endothelium is not absolute but involves “make and break” contacts which slows the neutrophil and allows it to “roll” across

the endothelium (Goetz *et al.* 1994). Vascular permeability is increased in the presence of factors such as complement component C5a and leukotriene B4 (LTB4) (DiStasi & Ley 2009) allowing the neutrophil to transmigrate between the endothelial cells (diapedesis; Kvietys & Sandig 2001). Neutrophil diapedesis is facilitated by the characteristic lobulated nucleus and the actin cytoskeleton thereby enabling flexible movement. Extravasated neutrophils are then recruited to sites of tissue injury or infection in response to chemotactic gradients present within the periodontal tissues including the chemotaxins CXCL8 (IL-8), fMLP and MIP-1 $\alpha$ .

### **1.5.3 Neutrophil chemotaxis**

Once neutrophils are recruited into the surrounding tissues, chemokines such as CXCL8 (IL-8) as well as bacterial products such as fMLP, create a chemotactic gradient essentially by diffusion away from the site of production. This directs host inflammatory-immune cells, including neutrophils, to the site of inflammation via chemotaxis (Kolaczowska & Kubes 2013). Neutrophil chemotaxis involves several stages which, to date, are relatively poorly understood but initially involve the binding of chemoattractants to specific cell surface receptors. Bacterial formylpeptides such as fMLP are one of the major chemotactic stimuli (Maney & Walters 2009) and neutrophils typically express approximately 55,000 G-protein-coupled formylpeptide receptors (FPRs) per cell (Pike & Snyderman 1988, Maney & Walters 2009) which, when activated, initiate a cascade of enzymatic reactions resulting in cell polarisation, orientation and migration towards the source of the chemoattractant (Snyderman & Goetzl 1981, Springer 1995, Katanaev 2001, Yagi *et al.* 2009). Activation of phosphoinositide 3-kinase (PI<sub>3</sub>K) (Hawkins *et al.* 2006) following signal transduction with phosphoinositide-dependent kinase (PDK1) (Yagi *et al.* 2009) leads to the accumulation of phosphatidylinositol (3,4,5)-triphosphate (PIP<sub>3</sub>) along the leading edge of the migrating neutrophil thereby regulating cell orientation (Andrew & Insall 2007, Yagi *et al.* 2009). This is followed by rapid morphological changes from round, relatively smooth cells to elongated ruffled cells with the formation of pseudopodia including a frontal protrusion which effectively pulls the neutrophil forward in a crawling motion. This is based on the reversible assembly of plasma membrane-associated microfilamentous cytoskeletal structures including monomeric globular G-actin conversion to polymerised filamentous F-actin (Stossel 1993, Champagne *et al.* 1998). The kinetics of actin polymerisation-depolarisation determines shape



changes that occur in the plasma membrane-associated cytoskeleton to provide the driving force for directional cell migration (Champagne *et al.* 1998).

Cytokine	Stimuli	Reference	Functions	Reference
<b>IL-1<math>\alpha</math></b>	HAP	Velard <i>et al.</i> 2009	Neutrophil chemotaxis ↑ neutrophil ROS	Lee <i>et al.</i> 2011 Yagisawa <i>et al.</i> 1995
<b>IL-1ra</b>	LPS Y-IgG / fMLP GM-CSF / TNF- $\alpha$	Muzio <i>et al.</i> 1994 Cassatella <i>et al.</i> 1997 McColl <i>et al.</i> 1992	IL-1 receptor antagonist	Ruth <i>et al.</i> 1996
<b>IL-1<math>\beta</math></b>	Unstimulated/GM-CSF LPS Casein	Galbraith <i>et al.</i> 1997 Jenkins <i>et al.</i> 1994 Goto <i>et al.</i> 1989	Neutrophil chemotaxis ↑ hepatic acute-phase protein	Oliveira <i>et al.</i> 2008 Gabay <i>et al.</i> 2001
<b>IL-3</b>	Ionomycin / PMA	Kato <i>et al.</i> 1990	Haemopoietic growth factor	Korpelainen <i>et al.</i> 1996
<b>IL-6</b>	Unstimulated LPS	Melani <i>et al.</i> 1993 Hatanaka <i>et al.</i> 2006	↑ hepatic acute-phase protein ↑ osteoclast activity ↑ B-cell differentiation ↑ T-cell activation Neutrophil chemotaxis ↑ ICAM-1 induction	Heinrich <i>et al.</i> 1990 Adebanjo <i>et al.</i> 1998 Maeda <i>et al.</i> 2010 Kishimoto 1989 Fielding <i>et al.</i> 2008 Wung <i>et al.</i> 2005
<b>IL-10</b>	Unstimulated <i>C. albicans</i>	Ocuin <i>et al.</i> 2011 Romani <i>et al.</i> 1997	↓ neutrophil ROS ↓ T-cell proliferation ↓ APC signalling receptors	Dang <i>et al.</i> 2006 Scott <i>et al.</i> 2006 Menezes <i>et al.</i> 2009
<b>IL-12</b>	<i>C. albicans</i> LPS <i>T. gondii</i> Herpes simplex virus	Romani <i>et al.</i> 1997 Cassatella <i>et al.</i> 1997 Bliss <i>et al.</i> 2000 Kanangat <i>et al.</i> 1996	↑ Th1 responses	Hsieh <i>et al.</i> 1993
<b>TNF-<math>\alpha</math></b>	Unstimulated LPS Y-IgG fMLP	Galbraith <i>et al.</i> 1997 Dubravec <i>et al.</i> 1990 Bazzoni <i>et al.</i> 1991 Cassatella <i>et al.</i> 1997	↑ ICAM-1 expression ↑ neutrophil ROS ↑ neutrophil degranulation	Wung <i>et al.</i> 2005 Chen <i>et al.</i> 2006 Brandt <i>et al.</i> 1992

<b>TGF-<math>\beta</math></b>	LPS	Grotendorst <i>et al.</i> 1989	Neutrophil chemotaxis ↑ neutrophil phagocytosis & ROS ↑ collagen synthesis and repair	Parekh <i>et al.</i> 1994 Lagraoui & Gagnon 1997 Duncan <i>et al.</i> 1999
<b>GM-CSF</b>	Ionomycin PMA	Kato <i>et al.</i> 1990	Haemopoietic growth factor Neutrophil chemotaxis ↑ neutrophil apoptosis ↑ neutrophil degranulation ↑ neutrophil ROS ↑ ICAM-1 expression	Fossati <i>et al.</i> 1998 Gomez-Cambronero <i>et al.</i> 2003 Kobayashi <i>et al.</i> 2005 Kaufman <i>et al.</i> 1989 Matthews <i>et al.</i> 2007 Bober <i>et al.</i> 1995
<b>IFN-<math>\alpha</math></b>	G-CSF PMA	Shirafuji <i>et al.</i> 1990 Denny <i>et al.</i> 2010	↑ T-cell proliferation ↑ neutrophil ROS ↑ NET release	Hervas-Stubbs <i>et al.</i> 2011 Wright <i>et al.</i> 2008 Martinelli <i>et al.</i> 2004
<b>IFN-<math>\gamma</math></b>	IL-12 TNF- $\alpha$	Ethuin <i>et al.</i> 2004 Yeaman <i>et al.</i> 1998	Neutrophil chemotaxis ↑ neutrophil ROS ↑ NET release	Jeppson <i>et al.</i> 1991 Wright <i>et al.</i> 2008 Martinelli <i>et al.</i> 2004
<b>OSM</b>	LPS GM-CSF / PMA	Greiner <i>et al.</i> 1999	Haemopoietic growth factor Modulate cell differentiation	Wallace <i>et al.</i> 1995
<b>HGF</b>	Unstimulated fMLP / GM-CSF TNF- $\alpha$	Wislez <i>et al.</i> 2003	↑ angiogenesis ↑ epithelial cell proliferation	Grant <i>et al.</i> 1993 Weidner <i>et al.</i> 1993

**Table 1.1. Known cytokines released by neutrophils *in vitro*, including recognised stimuli and predominant functions.**

Abbreviations: ↑, increase; IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; PG, prostaglandin; ROS, reactive oxygen species; Y-IgG, IgG-opsonised yeast (*Saccharomyces cerevisiae*); LPS, E-coli lipopolysaccharide; GM-CSF, granulocyte/macrophage colony stimulating factor; PMA, phorbol-12-myristate-13-acetate; HAP, hydroxyapatite; OSM, oncostatin M; HGF, hepatocyte growth factor; APC, antigen presenting cell; NET, neutrophil extracellular trap.

<b>Chemokine</b>	<b>Stimuli</b>	<b>Reference</b>	<b>Function</b>	<b>Reference</b>
<b>IL-8 (CXCL8)</b>	Unstimulated LPS <i>P. gingivalis</i> <i>A. actinomycetemcomitans</i> Zymosan A TNF- $\alpha$ IL-1 $\beta$ IL-12 fMLP GM-CSF Y-IgG C3a C5a LTB4 HAP <i>C. neoformans</i>	Fredriksson <i>et al.</i> 2002 Hatanaka <i>et al.</i> 2006  Restaino <i>et al.</i> 2007  Hattar <i>et al.</i> 2001 Ogilvie <i>et al.</i> 1996 Ethuin <i>et al.</i> 2001 Cassatella <i>et al.</i> 1997 Kurt-Jones <i>et al.</i> 2002 Cassatella <i>et al.</i> 1997  Vecchiarelli <i>et al.</i> 1998  McCain <i>et al.</i> 1994 Velard <i>et al.</i> 2009 Vecchiarelli <i>et al.</i> 1998	$\uparrow$ neutrophil chemotaxis $\uparrow$ neutrophil phagocytosis $\uparrow$ neutrophil ROS $\uparrow$ NET release	Hammond <i>et al.</i> 1995 Baggiolini <i>et al.</i> 1989 Guichard <i>et al.</i> 2005 Brinkmann <i>et al.</i> 2004
<b>GRO-<math>\alpha</math></b>	LPS TNF- $\alpha$ Y-IgG GM-CSF fMLP	Gasperini <i>et al.</i> 1995   Cassatella <i>et al.</i> 1999	$\uparrow$ neutrophil chemotaxis $\uparrow$ neutrophil ROS	Geiser <i>et al.</i> 1993 Oppenheim <i>et al.</i> 1991
<b>GRO-<math>\beta</math></b>	LPS	Iida & Grotendorst 1990	$\uparrow$ neutrophil chemotaxis / ROS	Magazin <i>et al.</i> 1992
<b>IP-10</b>	IFN $\gamma$ IFN $\gamma$ & LPS IFN $\gamma$ & TNF- $\alpha$ IFN- $\alpha$ & TNF- $\alpha$ IFN- $\alpha$ & LPS IFN- $\alpha$ & IL-1 $\beta$	Cassatella <i>et al.</i> 1997   Gasperini <i>et al.</i> 1999	$\uparrow$ T-cell chemotaxis	Taub <i>et al.</i> 1993

<b>MIG</b>	IFN $\gamma$ IFN $\gamma$ & LPS IFN $\gamma$ & TNF- $\alpha$	Gasperini <i>et al.</i> 1999	↑ T-cell chemotaxis	Mazanet <i>et al.</i> 2000
<b>I-TAC</b>	IFN $\gamma$ IFN $\gamma$ & LPS IFN $\gamma$ & TNF- $\alpha$	Gasperini <i>et al.</i> 1999	↑ T-cell chemotaxis	Cole <i>et al.</i> 1998
<b>MIP-1<math>\alpha</math></b>	HAP LPS LPS TNF- $\alpha$	Velard <i>et al.</i> 2009 Kasama <i>et al.</i> 1993 Cassatella <i>et al.</i> 1999	↑ neutrophil chemotaxis ↑ neutrophil ROS	Wolpe <i>et al.</i> 1988
<b>MIP-1<math>\beta</math></b>	LPS TNF- $\alpha$	Cassatella <i>et al.</i> 1999	↑ T-cell chemotaxis	Taub <i>et al.</i> 1993
<b>MCP-1</b>	HAP IFN $\gamma$ IFN $\gamma$ & LPS	Velard <i>et al.</i> 2009 Yoshimura <i>et al.</i> 2007	↑ monocyte chemotaxis	Yoshimura <i>et al.</i> 2007
<b>CINC-1</b>	Unstimulated	Cassatella <i>et al.</i> 1999	↑ neutrophil chemotaxis	Shibata <i>et al.</i> 1995
<b>CINC-2<math>\alpha</math></b>	Unstimulated	Cassatella <i>et al.</i> 1999	↑ neutrophil chemotaxis	Shibata <i>et al.</i> 1995

**Table 1.2. Known chemokines released by neutrophils *in vitro*, including recognised stimuli and predominant functions.**  
Abbreviations: ↑, increase; ROS, reactive oxygen species; IL, interleukin; Y-IgG, IgG-opsonised yeast (*Saccharomyces cerevisiae*); LPS, *E-coli* lipopolysaccharide; GM-CSF, granulocyte/macrophage colony stimulating factor; GRO- $\alpha$ , growth-related oncogene- $\alpha$ ; IP-10, interferon- $\gamma$ -inducible protein-10; MIG, monokine induced IFN $\gamma$ ; I-TAC, IFN-inducible T cell chemoattractant; MIP-1, macrophage inflammatory protein 1; MCP-1, monocyte chemoattractant protein-1; CINC-1, cytokine-induced neutrophil chemoattractant 1; HAP, hydroxyapatite; NET, neutrophil extracellular trap.

#### **1.5.4 Neutrophil antimicrobial response**

Neutrophils have various antimicrobial mechanisms with which to combat invading bacteria. These include phagocytosis, intra- and extracellular release of reactive oxygen species (ROS), antimicrobial enzymes and granule proteins, along with the release of neutrophil extracellular traps (NETs).

##### **1.5.4.1 Phagocytosis**

Bacterial binding by neutrophil cell surface receptors (e.g. the majority of toll-like receptors/TLRs, namely TLR1-10 except TLR3; Hayashi *et al.* 2003) activate phagocytosis causing the neutrophil to engulf the microorganism by sending out pseudopodia and then internalising it within a phagocytic vacuole (phagosome) bound by the neutrophil cell membrane (Lee *et al.* 2003). Neutrophils may also recognise host-derived molecules such as antibody and complement bound to the bacterial surface (e.g. IgG, C3b) through opsonisation utilizing Fcγ and C3b receptors. During phagocytosis, the contents of the neutrophil granules (lysosomes) are released and fuse with the phagosome to create a phagolysosome thereby creating a highly toxic microenvironment (Nordenfelt & Tapper 2011). Some targets may be too large to be fully phagocytosed or they avoid engulfment resulting in “frustrated phagocytosis” in which no phagosome is formed, but may then be killed extracellularly either with the extracellular release of ROS, granule proteins or NETs. These latter mechanisms however are associated with collateral host tissue damage.

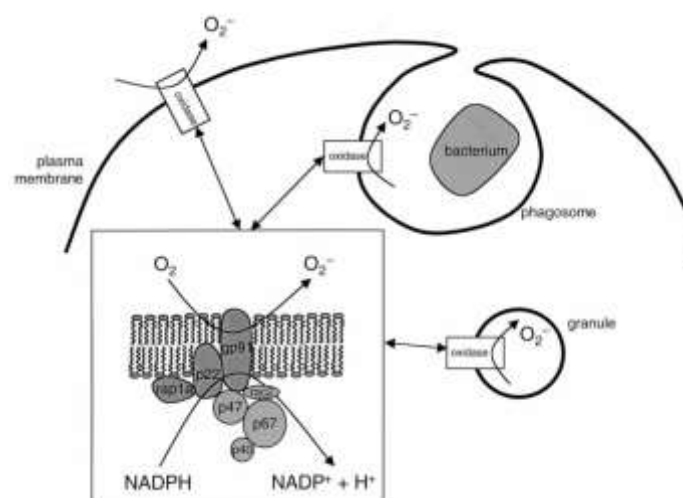
##### **1.5.4.2 Neutrophil antimicrobial activity**

Neutrophils display antimicrobial activity by both oxidative (oxygen-dependent) and non-oxidative (oxygen-independent) methods. Non-oxidative antimicrobial mechanisms involve the release of proteases and antimicrobial peptides contained within the azurophilic (primary), specific (secondary) and gelatinase (tertiary) neutrophil granules (Faurschou & Borregaard 2003, Nussbaum & Shapira 2011) which are formed sequentially during granulocyte differentiation (Borregaard 1995, 1997, 2010). In contrast, the oxidative killing mechanisms employed by neutrophils are the result of the respiratory burst (Babior 1973), with assembly of the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase responsible for the production of superoxide and subsequently downstream ROS including hydrogen peroxide and hypochlorous acid. Control and modulation of ROS activity is normally

achieved by antioxidants that significantly delay or prevent oxidation of substrates (Halliwell 1995, Chapple *et al.* 1997). Extracellular antioxidants include ascorbate,  $\alpha$ -tocopherol, carotenoids, metal binding proteins and compounds with oxidisable thiol groups such as glutathione (GSH; Chapple 1996, 2013, Waddington *et al.* 2000, Brock *et al.* 2004). The importance of ROS to the function of neutrophils is demonstrated in chronic granulomatous disease (CGD). This is a genetic disorder resulting in a dysfunctional NADPH oxidase, and therefore failure to generate ROS, with individuals subsequently susceptible to frequent and life-threatening bacterial and fungal infections (Dinauer 2005).

#### 1.5.4.3 Production of reactive oxygen species

Production of neutrophil superoxide involves activation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase which, together with molecular oxygen, comprises the neutrophil “respiratory burst” (Babior 1999, Dahlgren *et al.* 1999). NADPH-oxidase has a complex structure including four cytosolic subunits ( $p40^{\text{phox}}$ ,  $p47^{\text{phox}}$ ,  $p67^{\text{phox}}$  and Rac) that once phosphorylated enable combination with membrane-bound cytochrome b558 (comprised of  $p22^{\text{phox}}$ ,  $gp91^{\text{phox}}$ ) (Chapple & Matthews 2007). Once the NADPH-oxidase complex is fully assembled at the vacuolar membrane, it generates superoxide anions ( $O_2^-$ ) by single-electron reduction of molecular oxygen (Babior 1973, Segal 1989) prior to subsequent conversion to downstream ROS such as, hydrogen peroxide and hypochlorous acid (Figure 1.3).



**Figure 1.3. NADPH oxidase assembly and activity in human neutrophils** (Dahlgren & Karlsson 1999).

Superoxide is subsequently released into the phagosomal space and also into the extracellular environment at a rate of 5-10mmol/l/s (Hampton *et al.* 1998, Waddington 2000). Superoxide is relatively short-lived and dismutates to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) either spontaneously or via the enzyme superoxide dismutase (SOD) (McCord & Fridovich 1969, Battino *et al.* 1999). Hydrogen peroxide is capable of diffusing across cell and nuclear membranes and may be released extracellularly. Alternatively, intracellular hydrogen peroxide is rapidly catabolised by catalase or glutathione peroxidase. Although  $\text{H}_2\text{O}_2$  alone is microbicidal, its antimicrobial potential is further enhanced by its conversion to downstream ROS in the presence of peroxidase enzymes such as myeloperoxidase (MPO) which constitutes ~5% of the human neutrophil by weight and is responsible for oxidizing halides (Nauseef 1983). Chlorine is usually oxidized in the presence of  $\text{H}_2\text{O}_2$  because of the relatively high concentration within host tissues, resulting in the formation of hypochlorous acid (HOCl) (Nauseef *et al.* 1983). In addition,  $\text{H}_2\text{O}_2$  may also undergo a 'Fenton reaction' in the presence of  $\text{Fe}^{2+}$  or  $\text{Cu}^{2+}$  forming a potent hydroxyl radical ( $\cdot\text{OH}$ ; Sutton & Winterbourne 1989). This is important given that the formation of  $\cdot\text{OH}$  is site-specific, since uncharged  $\text{H}_2\text{O}_2$  diffuses across lipid membranes and is subsequently converted to the  $\cdot\text{OH}$  radical in close proximity to vital cell structures such as DNA. Whilst soluble ferrous iron ( $\text{Fe}^{2+}$ ) is not normally present *in vivo*, being bound tightly to proteins, it can be produced within cells (e.g. mitochondria or cytosol) by the action of  $\text{O}_2^-$  on ferric iron ( $\text{Fe}^{3+}$ ) within iron storage proteins.

#### **1.5.4.4 Function of reactive oxygen species**

With regards to host defence, including the pathogenesis of chronic periodontitis, arguably one of the most important functions of ROS relate to their antimicrobial activity as reviewed by Chapple & Matthews (2007). The superoxide radical and  $\text{H}_2\text{O}_2$  alone have relatively low antimicrobial activity and toxicity in comparison to that observed following oxidase activation, and unless concentrations exceed 50 $\mu\text{M}$  the cytotoxicity of  $\text{H}_2\text{O}_2$  is limited (Halliwell *et al.* 2000). Similarly, HOCl formed from  $\text{H}_2\text{O}_2$  (section 1.5.4.3) is directly cytotoxic provided that concentrations exceed 20 $\mu\text{M}$  (Mainemare *et al.* 2004). The antimicrobial activity of neutrophil ROS also relates to indirect activation of lysosomal proteases. This has been shown to occur with neutrophil activation raising intra-cellular  $\text{Ca}^{2+}$  concentrations causing subsequent opening of  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels. Concurrent  $\text{O}_2^-$  release results in membrane depolarisation thereby creating an ionic gradient with activation



of antimicrobial lysosomal proteases within a hypertonic  $K^+$ -rich alkaline environment (Reeves *et al.* 2002). HOCl is also an important trigger with regard to subsequent NET release which is an additional antimicrobial neutrophil defence strategy (Palmer *et al.* 2012; section 1.5.4.5).

In addition to antimicrobial activity, ROS production is vital to a broad range of diverse pro-inflammatory biological activities with the different subtypes of ROS having different effects on both bacterial and host cells (reviewed by Chapple & Matthews 2007). For example, the production of  $O_2^-$  from the NADPH oxidase activates host cell NF- $\kappa$ B with subsequent transcription of pro-inflammatory cytokines (Ndengele *et al.* 2005), as well as increasing vascular permeability (Haglund *et al.* 1994) and neutrophil chemotaxis via LTB<sub>4</sub> formation (Deitch *et al.* 1990). In addition,  $O_2^-$  has the potential to cause endothelial cell damage (Droy-Lefaix *et al.* 1991), lipid peroxidation (Dix & Aikens 1993) and DNA strand breaks (Dix *et al.* 1996). Subsequent formation of  $H_2O_2$  acts primarily as a second messenger in NF- $\kappa$ B activation (Halliwell *et al.* 2000) and has varying biological functions including increasing adhesion molecule expression (Roebuck 1999), inducing apoptosis (Clement *et al.* 1998) and also modulating platelet aggregation (Naseem & Bruckdorfer 1995). In addition, the  $\cdot OH$  radical and HOCl (section 1.5.4.4) are regarded as the most potent ROS species and known to cause cell and tissue damage including lipid peroxidation (Mylonas & Kouretas 1999), protein damage (Fu *et al.* 1995), DNA and RNA damage (Dix *et al.* 1996) as well as the oxidation of important antiproteases such as  $\alpha$ 1-antitrypsin (Mohsenin & Gee 1989) and degradation of extracellular matrix components (Waddington *et al.* 2000). With regard to the pathogenesis of chronic periodontitis,  $O_2^-$  and  $H_2O_2$  have been shown to activate osteoclasts (Bax *et al.* 1992, Hall *et al.* 1995), promote osteoclast formation (Garrett *et al.* 1990) and also degrade alveolar bone proteoglycans (Moseley *et al.* 1998). In addition, HOCl has been shown to degrade type I collagen from the periodontal ligament via oxidative degradation (Petersen *et al.* 2004), with neutrophil-generated HOCl also being shown to directly cause epithelial cell lysis (Altman *et al.* 1992).

Given the different antimicrobial and biological functions of ROS, their imbalanced release leading to oxidative stress is regarded as being responsible (both directly and indirectly) for causing collateral host-mediated tissue damage in the pathogenesis of chronic periodontitis as

well as other systemic diseases such as rheumatoid arthritis (reviewed by Chapple & Matthews 2007; section 1.3). Interestingly, a deficiency in the key antioxidant reduced glutathione (GSH) has been shown in GCF in periodontitis patients (Chapple *et al.* 2002, Grant *et al.* 2010) and also in peripheral blood neutrophils. In the latter situation the redox state of the neutrophil (GSH:GSSG ratio) was shown to be reversed leading to cellular stress that resulted in relocation of NADPH oxidase to the outer cell membrane (Dias *et al.* 2013). This may explain the excess extracellular ROS release reported by Matthews *et al.* 2007 (neutrophil hyperactivity) and associated potential collateral tissue damage.

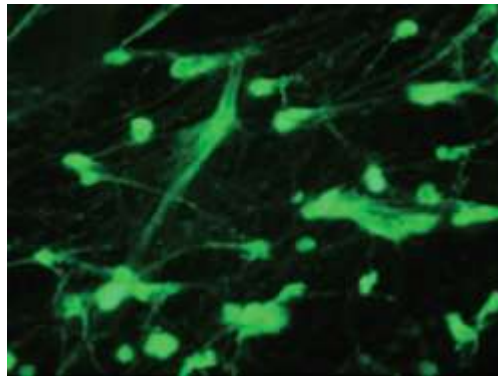
#### **1.5.4.5 Neutrophil extracellular traps**

As well as the production of ROS, neutrophils also have the ability to release strand-like webs of decondensed nuclear chromatin containing granule proteins that function to immobilise and potentially kill invading bacteria. This process, involving the release of neutrophil extracellular traps (NETs) is regarded as being as powerful an antimicrobial strategy as that of phagocytosis (Brinkmann *et al.* 2004, Fuchs *et al.* 2007). Neutrophil extracellular traps consist of histones and nuclear DNA wrapped as fibres of approximately 30nm in diameter that are decorated with globular antimicrobial proteins (e.g. neutrophil elastase, myeloperoxidase) that originate from the neutrophil granules (Brinkmann *et al.* 2007, Urban 2009; Figures 1.4, 1.5).

Interestingly, histones are one of the oldest known families of human proteins and it has long been established that whilst their primary function is to package DNA as compactly as possible they also have the potential to bind DNA to form a nucleosome complex with antimicrobial activity. The biological significance of this observation was initially unclear as it was not understood where or how histones would encounter microorganisms during an infection (Hirsch 1958). However, histones have since been shown to account for ~70% of the total protein content of NETs (Urban *et al.* 2009), the discovery of which now highlights that histones are active outside the chromatin milieu and no longer restricted to the nuclear environment (Parseghian 2006).



**Figure 1.4. Scanning electron micrographs of neutrophils with associated fibres from extruded neutrophil extracellular traps (Cooper *et al.* 2013).**



**Figure 1.5. Fluorescence photomicrograph of neutrophil extracellular traps stained with the DNA dye Sytox green (Palmer *et al.* 2012).**

Extracellular trap release is not solely confined to neutrophils (Brinkman *et al.* 2004), and is also associated with mast cells (von Kockritz-Blickwede *et al.* 2009) and eosinophils (Yousefi *et al.* 2008). Extracellular trap release is now considered a fundamental mechanism of the innate immune response and has been observed *in-vivo* in humans (Brinkman *et al.* 2004; Gupta *et al.* 2005), mice (Beiter *et al.* 2006; Buchanan *et al.* 2006; Wartha *et al.* 2007), rabbits (Brinkman *et al.* 2004), horses (Alghamdi *et al.* 2005), cows (Lippolis *et al.* 2006) and fish (Palic *et al.* 2007).

Currently recognised stimuli for NET release include HOCl (Palmer *et al.* 2012), nitric oxide (Keshari *et al.* 2012), IL-8 (Brinkmann *et al.* 2004), bacterial toxins (Brinkmann *et al.* 2004, Fuchs *et al.* 2007, Landoni *et al.* 2012, Mori *et al.* 2012, Revelo & Waldron 2012), yeasts (Urban *et al.* 2006), protozoa (Guimaraes-Costa *et al.* 2009, Behrendt *et al.* 2010, Abi Abdallah *et al.* 2012), antimicrobial peptides (Kraemer *et al.* 2011), antibodies (Kessenbrock

*et al.* 2009, Lande *et al.* 2011, Thomas *et al.* 2012), platelets (Clark *et al.* 2007, Massberg *et al.* 2010, Caudrillier *et al.* 2012, von Bruhl *et al.* 2012) and statins (Chow *et al.* 2010). It has also been suggested that activation of multiple receptors is required for optimal stimulation of NETs (Fuchs *et al.* 2007), along with cytokine priming (Yousefi *et al.* 2009). Release of NETs by neutrophils can also be induced by phorbol 12-myristate 13-acetate (PMA), a non-physiological and direct activator of protein kinase C (PKC) that is independent of membrane receptor-ligand interaction and phagocytosis. Thus, PMA is widely used as a positive-control stimulus for NET formation (Fuchs *et al.* 2007).

#### **1.5.4.6 Formation of neutrophil extracellular traps**

Formation of NETs is dependent upon HOCl production via NADPH oxidase (Fuchs *et al.* 2007, Remijnsen *et al.* 2011, Akong-Moore *et al.* 2012, Palmer *et al.* 2012) and hyper-citrullination of histones by peptidylarginine deiminase 4 (PAD4), which converts arginine and methylarginine residues of histones to citrulline. Interestingly, a PAD enzyme (PPAD) has been shown to be released from the periodontal pathogen *P. gingivalis* (McGraw *et al.* 1999, Bingham & Moni 2013). Citrullination enables decondensation and unfolding of the DNA/chromatin complex (Gyorgy *et al.* 2006, Wang *et al.* 2009, Li *et al.* 2010) which then expands to the inner margins of the nuclear membrane. This causes the nucleus to swell and lose its lobules and form distinct vesicles (Fuchs *et al.* 2007, Borregaard *et al.* 2010). Neutrophil granules and nuclear membranes are then lost, allowing the release and mixing of decondensed chromatin and granule proteins, particularly neutrophil elastase (Fuchs *et al.* 2007). Interestingly, plasma membrane integrity of activated neutrophils is not affected by the disruption of internal membranes, allowing the intracellular mixing of NET components, before the eventual rupture and loss of the outer membrane integrity and subsequent loss of cytoplasm (Fuchs *et al.* 2007). Active extrusion of the extracellular trap complex involves the actin cytoskeleton and microtubular complex (Brinkmann *et al.* 2004, Guimaraes-Costa *et al.* 2009, Lauth *et al.* 2009, Neeli *et al.* 2009, Aulik *et al.* 2010, Abi Abdallah *et al.* 2012, Palmer *et al.* 2012). Although NET formation was initially regarded as a terminal event, albeit different to necrosis and apoptosis (Brinkmann *et al.* 2004, Fuchs *et al.* 2007), NETs comprising mitochondrial rather than nuclear DNA have also been shown and released from viable neutrophils (Yousefi *et al.* 2009).

#### **1.5.4.7 Function of neutrophil extracellular traps**

Neutrophil extracellular traps have been shown to be effective in killing Gram negative (Grinberg *et al.* 2008) and Gram positive bacteria (Brinkmann *et al.* 2004), parasites (Abi Abdallah *et al.* 2012) and fungi (Urban *et al.* 2006). The antimicrobial actions of NETs are twofold: firstly, the trapping and immobilising of pathogens preventing tissue and systemic spread, and secondly pathogen killing by NET-embedded cathelicidin antimicrobial peptides (Cooper *et al.* 2013). Whilst phagocytosis is the preferred method of bacterial killing by neutrophils, NETs can act as a secondary killing approach if required (Fuchs *et al.* 2007). It is also possible that NETs also sequester cytokines and chemokines in the local environment of the neutrophil and thereby minimise untoward dissemination of the inflammatory response thereby potentially minimising damage to the surrounding host tissues (Bank & Ansorge 2001).

### **1.6 NEUTROPHIL FUNCTION IN CHRONIC PERIODONTITIS**

Neutrophils are the predominant cell within the gingival crevice and pocket epithelium (Gustafsson & Asman 1996, Nussbaum *et al.* 2011), constituting ~50% of the leukocytes infiltrating the junctional epithelium and ~90% of the leukocytes isolated from GCF (Miyasaki *et al.* 1991). Rather than forming an homogenous bacteria/leukocyte mixture in the gingival crevice, neutrophils have been shown to form a “leukocyte wall” interposed between the periodontal plaque mass and the junctional and sulcular (or pocket) epithelium (Attstrom 1971). Immunocytochemical analyses of inflamed periodontal tissues have also confirmed the presence of large numbers of recruited neutrophils in diseased tissue *in vivo* (Hou *et al.* 2003). Neutrophils are considered central to the pathogenesis of chronic periodontitis, with both hypo- and hyper-responsive phenotypes having the potential to result in collateral periodontal host tissue damage. A hypo-responsive neutrophil will be unable to effectively manage the presence of periodontal bacteria. However a hyper-responsive neutrophil could equally result in periodontal tissue destruction as a result of collateral host tissue damage through inappropriate release of antimicrobial factors such as ROS, NETs and antimicrobial peptides, along with the potential to further propagate the chronic inflammatory lesion by the inappropriate release of cytokines within the surrounding tissues and systemic circulation. Therefore, a delicate balance exists with regards to neutrophil function in chronic periodontitis, and excessive ROS release and oxidative stress is considered central to the

pathogenesis of chronic periodontitis (reviewed by Chapple & Matthews 2007). Furthermore, ROS-induced exaggerated NET release will result in increased extracellular DNA which may breach host immunotolerance and result in the generation of autoantibodies, which may also play a role in the pathogenesis of chronic periodontitis (Cooper *et al.* 2013).

### **1.6.1 Neutrophil cytokine release in chronic periodontitis**

The majority of studies investigating the presence of cytokines in chronic periodontitis have been performed on plasma, GCF and tissue biopsy samples and generally show higher levels of cytokines in disease compared to health. Greater plasma concentrations of IL-8 (Fredrikson *et al.* 2003, Dias *et al.* 2011), IL-6 (Loos *et al.* 2000, Monteiro *et al.* 2009, Lappin *et al.* 2011, Gani *et al.* 2012) and IL-1 $\beta$  (Howells 1995, Sadik *et al.* 2012) have been demonstrated in patients with chronic periodontitis. Similarly, increased GCF concentrations of IL-8 (Lagdive *et al.* 2013), IL-6 (Fagundes *et al.* 2011), IL-1 $\beta$  (Howells 1995, Figueredo *et al.* 1999) and TNF- $\alpha$  (Drugarin *et al.* 1998, Galmonal *et al.* 2003, Fagundes *et al.* 2011) have also been reported in chronic periodontitis patients compared to healthy controls. Interestingly, IL-6 and IL-1 $\beta$  concentrations in saliva have also been shown to increase in a 21-day experimental gingivitis model with levels increasing as plaque accumulated, indicating that cytokine concentrations in saliva reflect the degree of gingival inflammation (Zhou *et al.* 2012). Immunohistochemical analyses of inflamed gingival tissue samples, obtained from patients with chronic periodontitis, also demonstrate elevated *in-vivo* IL-8 (Liu *et al.* 2001), IL-1 $\beta$  (Feldner *et al.* 1994, Lo *et al.* 1999, Hou *et al.* 2003) and TNF- $\alpha$  (Liu *et al.* 2001).

To date, there have only been 4 published studies investigating peripheral blood neutrophil cytokine release from patients with chronic periodontitis (Galbraith *et al.* 1997, Figueredo *et al.* 2000, Fredriksson *et al.* 2002, Restaino *et al.* 2007). Such studies may be of importance in terms of assessing the relative contribution of neutrophil cytokine release with regards to the reported increased cytokine levels observed in chronic periodontitis. Two studies have demonstrated that peripheral blood neutrophils from periodontitis patients release similar levels of IL-8, IL-1 $\beta$  and TNF- $\alpha$  compared to healthy controls during a 5hr incubation period (Galbraith *et al.* 1997, Fredriksson *et al.* 2002). Galbraith *et al.* cultured isolated neutrophils in the presence of GM-CSF for up to 5hrs prior to collection and analysis of the supernatant by ELISA. Whilst TNF- $\alpha$  was not detected following 3-5hr culture in the presence of GM-

CSF, increased IL-1 $\beta$  was identified with increasing time of culture, with IL-1 $\beta$  concentrations of up to 86.7pg/10<sup>7</sup> neutrophils and with no differences between patient and control cells (Galbraith *et al.* 1997). Interestingly, the same study was only able to identify TNF- $\alpha$  or IL-1 $\beta$  mRNA in stimulated but not unstimulated conditions at 3hrs (Galbraith *et al.* 1997). Fredriksson *et al.* cultured isolated neutrophils for 5hrs in the presence and absence of opsonised *S. aureus* prior to analysis of the supernatant by ELISA. Similarly no significant differences in IL-8 or TNF- $\alpha$  release were observed with median concentrations of  $\leq 9.8$  and  $\leq 36.7$ pg/ml (IL-8) and  $\leq 64.4$  and  $\leq 121.8$ pg/ml (TNF- $\alpha$ ) for unstimulated and Fc $\gamma$ R-stimulated conditions respectively (Fredriksson *et al.* 2002). By contrast, Restaino *et al.* demonstrated decreased IL-8 release from isolated neutrophils of patients with chronic periodontitis compared to healthy controls when cultured for 1hr in the presence and absence of different stimuli including LPS, phytohemagglutinin (PHA), zymosan A, *P. gingivalis* and *A. actinomycetemcomitans* (Restaino *et al.* 2007). Concentrations of ~800ng/ml were reported for healthy control neutrophils, with a lack of observable increased release in the presence of all stimuli investigated. However stimulation was confirmed for patient neutrophils with concentrations of ~100ng/ml and ~200ng/ml for unstimulated and stimulated cells respectively (Restaino *et al.* 2007). In contrast, flow cytometric analyses of fixed, permeabilised neutrophils has revealed significantly more IL-1 $\beta$  release in patients with chronic periodontitis compared to healthy controls (Figueredo *et al.* 2000).

Neutrophils are known to biosynthesize cytokines including IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-8 prior to their release (Lloyd & Oppenheim 1992) with cytokine synthesis induced following stimulation (Schindler *et al.* 1990, Cassatella 1999). Specific mechanisms of neutrophil cytokine release are currently poorly understood, and it is therefore important that sufficient neutrophil culture times are employed in order to measure suitable cytokine concentrations, along with increasing the likelihood of reproducible data. This is highlighted in the aforementioned studies whereby a lack of TNF- $\alpha$  release was observed within a 5hr incubation period, along with a lack of TNF- $\alpha$  and IL-1 $\beta$  mRNA (Galbraith *et al.* 1997). A lack of IL-8 stimulation above unstimulated conditions was also observed in the presence of periodontal pathogens within a 1hr culture period (Restaino *et al.* 2007). Longer culture times taking into consideration neutrophil half-life, will be more likely to reflect maximal cytokine release. *Escherichia coli* LPS represents the most commonly used, and therefore relatively

established, stimulus for neutrophil cytokine release (Table 1.1) with only one study having employed known periodontal pathogens (Restaino *et al.* 2007). In addition, none of the published papers investigating neutrophil cytokine release in chronic periodontitis specifically state post-culture viability testing, and this is important in order to distinguish active cytokine release from viable neutrophils in comparison to that released as a product of cell necrosis.

Type-2 diabetes displays a unique bi-directional relationship with chronic periodontitis (section 1.3), and peripheral blood neutrophils isolated from diabetes patients have recently been shown to release greater amounts of IL-8, IL-1 $\beta$  and TNF- $\alpha$  when cultured for 18hr, in the presence and absence of 5 $\mu$ g/ml LPS, than cells isolated from healthy controls (Hatanaka *et al.* 2006). To date there are no similar studies investigating neutrophil cytokine release within an 18hr culture period from neutrophils isolated from patients with chronic periodontitis compared to matched healthy controls. Given the importance of cytokines in modulating and coordinating the inflammatory-immune response including neutrophil chemotaxis (Graves *et al.* 2003), priming for ROS (Gainet *et al.* 1998, Van Dervort *et al.* 1998) and NET release (Brinkmann *et al.* 2004, Wang *et al.* 2009), along with osteoclastic bone remodelling (Ishimi *et al.* 1990, Rucci *et al.* 2008), such differences will provide additional information regarding the pathogenesis of chronic periodontitis.

### **1.6.2 Neutrophil chemotaxis in chronic periodontitis**

The vast majority of studies investigating neutrophil chemotaxis have been conducted with juvenile/aggressive periodontitis patients (reviewed by Ryder 2010) rather than chronic periodontitis. To date there are only 6 published studies investigating peripheral blood neutrophil chemotaxis in chronic periodontitis (Van Dyke *et al.* 1980, McMullen *et al.* 1981, Altman *et al.* 1985, Mouynet *et al.* 1994, Kumar & Prakash 2012, Srinivas *et al.* 2012) as summarised (Table 1.3). These studies effectively measure uni-directional cell movement using traditional Boyden chambers and under agarose methods thereby quantifying end points of cell movement, recently termed “chemokinesis”, rather than directly observing and measuring individual cell movement that is more closely representative of “true chemotaxis” (Muinonen-Martin *et al.* 2010). Studies investigating neutrophil chemokinesis report conflicting and inconsistent results. Significantly, increased chemokinesis towards *E. coli* supernatant, fMLP and LPS-activated serum has been reported in patients with chronic



periodontitis (Van Dyke *et al.* 1980, McMullen *et al.* 1981). No differences in neutrophil chemokinesis have also been observed towards LPS-activated serum, fMLP and C5a (Altman *et al.* 1985, Mouynet *et al.* 1994). In contrast, decreased neutrophil chemokinesis has also been reported including in relation to casein (Kumar & Prakash 2012, Srinivas *et al.* 2012).

A novel direct visualisation chemotaxis chamber has recently been developed by colleagues at the University of Glasgow (Muinonen-Martin *et al.* 2010), that has subsequently been used to more accurately measure neutrophil chemotaxis in patients diagnosed with chronic obstructive pulmonary disease (COPD; Sapey *et al.* 2011). Similar experiments have not been performed with regards to neutrophil chemotaxis in chronic periodontitis patients, and doing so would provide more detailed and accurate information on true neutrophil chemotaxis in the pathogenesis of periodontitis.

Reference	Method	Chemoattractant	Comparison to healthy control
Van Dyke <i>et al.</i> 1980	Boyden chamber	LPS-activated serum fMLP <i>E.coli</i> culture supernatant	↑ ↑ ↑
McMullen <i>et al.</i> 1981	Boyden chamber	LPS-activated serum fMLP <i>E.coli</i> culture supernatant	↑ ↑ ↑
Altman <i>et al.</i> 1985	Boyden chamber	LPS-activated serum C5a fMLP	↔ ↔ ↔
Mouynet <i>et al.</i> 1994	Under agarose	LPS-activated serum fMLP	↔ ↔
Kumar <i>et al.</i> 2012	Boyden chamber	Not stated	↓
Srinivas <i>et al.</i> 2012	Under agarose	Casein	↓

**Table 1.3. Studies investigating neutrophil chemotaxis in chronic periodontitis, including methods, chemoattractant and comparison to healthy controls.** Abbreviations: ↑, increase; ↔, no difference; ↓, decrease; LPS, lipopolysaccharide; fMLP, formyl-methionyl-leucyl-phenylalanine.

### 1.6.3 Neutrophil reactive oxygen species release in chronic periodontitis

Studies of ROS generation by peripheral blood neutrophils in periodontal disease include a variety of patient groups, different pathways of activation and methods of ROS detection (last reviewed by Chapple & Matthews 2007). To date, the role of neutrophil-derived ROS in the pathogenesis of periodontal disease is not completely understood.

Peripheral blood neutrophils isolated from patients with chronic periodontitis have been shown to be hyperactive with regards to baseline extracellular ROS release and also following FcγR and TLR stimulation, with and without priming with *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Escherichia coli* lipopolysaccharide (LPS) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fredriksson *et al.* 2003, Matthews *et al.* 2007). In addition, total ROS release is higher from neutrophils isolated from patients with chronic periodontitis in comparison to healthy controls following stimulation with opsonized *Staphylococcus aureus*, complement-opsonised zymosan and phorbol 12-myristate 13-acetate (PMA) (Gustafsson & Asman 1996, Fredriksson *et al.* 1998, 1999, 2003, Zekonis & Zekonis 2004, Matthews *et al.* 2007). In addition, no differences in ROS release from periodontitis neutrophils in response to priming with TNF-α, LPS or fMLP has been reported (Gustafsson *et al.* 1997). To date, there has been only one published longitudinal study investigating the effect of periodontal treatment on neutrophil ROS responses (Matthews *et al.* 2007). This study demonstrated that successful periodontal therapy reduced the total FcγR-stimulated neutrophil hyper-reactivity, but did not alter unstimulated extracellular ROS hyperactivity, suggesting that both constitutive and reactive mechanisms underlie elevated neutrophil ROS generation in chronic periodontitis (Matthews *et al.* 2007). As discussed previously, this may be due to a constitutive deficiency in GSH synthesis and activation of sphingomyelinase generating lipid rafts with NADPH oxidase components embedded within the outer cell membrane (Dias *et al.* 2013).

There have only been 3 published studies investigating peripheral blood neutrophil ROS release specific to superoxide using lucigenin-enhanced chemiluminescence in chronic periodontitis (Guarnieri *et al.* 1991, Zekonis *et al.* 2003, Zilinskas *et al.* 2011). Baseline unstimulated peripheral blood neutrophil superoxide release was reported to be higher from cells isolated from patients compared to periodontally healthy controls (Guarnieri *et al.* 1991,

Zekonis *et al.* 2003, Zilinskas *et al.* 2011). Greater neutrophil superoxide release is also reported following stimulation with opsonized zymosan and *E. coli* LPS (Zekonis *et al.* 2003, Zilinskas *et al.* 2011), although no differences between patient and control cells were observed following stimulation with PMA (Guarnieri *et al.* 1991).

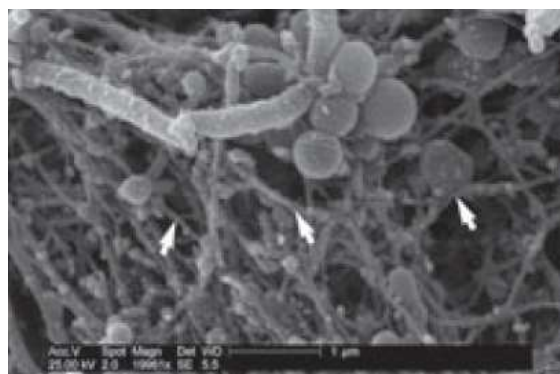
To date there have been no published studies investigating superoxide release from peripheral blood neutrophils isolated from patients with chronic periodontitis stimulated with periodontally-relevant bacteria, along with studying the effect of periodontal treatment. Such information is important in order to further characterize the hyperactive and –reactive neutrophil phenotype with regards to extracellular ROS release observed in chronic periodontitis and therefore for potential for collateral tissue damage.

#### **1.6.4 Neutrophil extracellular trap release in chronic periodontitis**

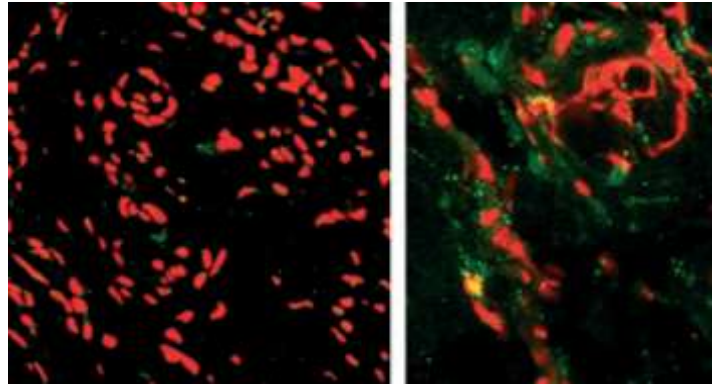
It has been hypothesized that periodontal disease pathogenesis may be associated with dysregulated NET release (Cooper *et al.* 2013). This is consistent with the fact that patients with chronic periodontitis exhibit a hyperactive/-reactive peripheral blood neutrophil phenotype in terms of ROS generation and release and that ROS are central to NET formation (Fuchs *et al.* 2007, Palmer *et al.* 2012). In addition, increased neutrophil ROS production has been associated with elevated type-I interferon (IFN $\alpha$ ) levels in periodontal disease (Wright *et al.* 2008) that have also been shown to prime for NET release (Martinelli *et al.* 2004). Whilst NETs are antimicrobial they are also thought to function to limit the untoward diffusion of degradative agents (e.g. proteases such as elastase) released from neutrophils (Bank & Ansorge 2001) and therefore locally contain the inflammatory-immune response. Although these functions may be beneficial to the host it has also been hypothesised that excessive NET production may be counter-productive (Lee & Grinstein 2004, Clark *et al.* 2007). For example, the presence of extracellular DNA-histones within NETs may act as a target for additional endogenous or bacterial-derived PADs with subsequent histone hypercitrullination creating autoimmunogenic epitopes thus stimulating the production of anti-citrullinated protein antibodies (ACPAs; Parseghian & Luhrs 2006). Importantly, *P. gingivalis*, regarded as one of the most important periodontal pathogens (Socransky *et al.* 1998), is the only bacteria capable of producing a PAD analogous to PAD4 and therefore capable of histone citrullination (Rosenstein *et al.* 2004). Therefore the activity of *Porphyromonas gingivalis*

PAD (PPAD) upon NET histones released into periodontal tissues may trigger a breach of immune tolerance via hypercitrullination, potentially adding an autoimmune component to periodontitis pathogenesis (Wright *et al.* 2008). This theory implies that anti-citrullinated protein antibodies (ACPA's) shall be detectable in non-rheumatoid arthritis patients with periodontitis, which was recently shown to be the case by our group (dePablo *et al.* 2013).

There are currently no published studies investigating NET release from peripheral blood neutrophils isolated from patients with chronic periodontitis. However *in-vivo* evidence of NETs in purulent exudates from the gingivae of chronic periodontitis patients has been reported (Vitkov *et al.* 2009) and considered comparable to those previously identified *in-vivo* in abscess exudates from group A *Streptococcus pyogenes* infections in mice (Buchanan *et al.* 2006), and also in a mixed bacterial infection of human appendicitis (Brinkmann *et al.* 2004). Transmission electron microscopy of periodontal pocket epithelium also reveals NETs associated with periodontal bacteria (Vitkov *et al.* 2009; Figure 1.6). Similarly, confocal image analysis has revealed for the first time the presence of NETs in inflamed but not healthy control gingival tissues (Cooper *et al.* 2013; Figure 1.7). Measurement of peripheral blood neutrophil NET release will provide additional novel information regarding this recently discovered antimicrobial defence mechanism, as well as providing information on the potential role of NETs in the pathogenesis of chronic periodontitis.



**Figure 1.6. Scanning electron microscopy of a periodontal pocket epithelium biopsy indicating the presence of neutrophil extracellular traps and entrapped bacteria (Vitkov *et al.* 2009).**



**Figure 1.7. The first demonstration of neutrophil extracellular trap structures within gingival connective tissues from a patient with gingivitis (right panel) compared with a healthy control (left panel).** Confocal images demonstrate the co-localisation of myeloperoxidase (in green) with DNA (in red) in gingivitis tissues where neutrophil extracellular trap-like structures are evident. In control tissues there are no neutrophil extracellular traps visible, simply nuclear DNA (Cooper *et al.* 2013).

## 1.7 C-REACTIVE PROTEIN

C-reactive protein (CRP) is a highly conserved protein that was originally discovered in Oswald Avery's laboratory at the Rockefeller Institute during studies of patients with *Streptococcus pneumoniae* infection (Tillet 1930). It is a member of the pentraxin family of proteins (Kathariya *et al.* 2013) due to its electron micrographic appearance from the Greek *penta* (five) and *ragos* (berries) (Pepys & Hirsch 2003). In its native form CRP is composed of five identical subunits, each containing 206 amino acid residues, non-covalently linked to form a symmetrical disc of approximately 115kDa (Pepys *et al.* 2003, Eisenhardt *et al.* 2009). More specifically, individual CRP subunit mass has been reported as 23,028Da by electrospray mass spectrometry, corresponding to the mass of 23,029Da predicted from the known amino acid sequence including the N-terminal pyroglutamic acid (Pepys *et al.* 2005, Oliveira *et al.* 1979).

In human plasma, CRP exists as a cyclic, disc-shaped pentamer of 115kDa as confirmed by electron microscopy, gel chromatography and crystallography (Osmand *et al.* 1977, Volkanis *et al.* 1978, Shrive *et al.* 1996, Thompson *et al.* 1999, Eisenhardt *et al.* 2009), however pentameric CRP can also undergo dissociation and aggregation under certain conditions, thereby acquiring distinct functionality (Ji *et al.* 2007).

C-reactive protein is predominantly produced by liver hepatocytes, primarily under transcriptional control by IL-6 (Gauldie *et al.* 1987), although other sites of local extra-hepatic CRP synthesis and secretion have also been identified including smooth muscle cells (Calabro *et al.* 2003), endothelial cells (Venugopal *et al.* 2005, Eisenhardt *et al.* 2009), neurons, atherosclerotic plaques, epithelial cells (Lu & Jin 2010), monocytes and lymphocytes (Jialal *et al.* 2004; Kuta *et al.* 1986). C-reactive protein has also been detected in all cell layers of inflamed and non-inflamed gingival tissue samples using immunohistochemistry, Western blotting and gene expression analysis and, as such, human gingivae have been postulated as being another site of CRP production (Lu & Jin 2010).

### **1.7.1 C-reactive protein concentrations in health & disease**

In healthy individuals the median concentration of CRP is ~0.8mg/l, but following an acute-phase stimulus values may increase significantly to  $\geq 500$ mg/l (Pepys & Hirschfield 2003). C-reactive protein, although non-specific, is regarded as the most sensitive indicator of systemic inflammation (Coimbra *et al.* 2009) and as such is widely used medically as a systemic inflammatory marker in the diagnosis of chronic infections and inflammatory conditions (Pepys & Hirschfield 2003, Ramamoorthy *et al.* 2012). C-reactive protein is also widely used as a predictor of cardiovascular disease risk with moderate increases being predictive of cardiovascular events in otherwise apparently healthy individuals (Ridker *et al.* 1997), with the American Heart Association guidelines for cardiovascular disease risk being 0.1, 1.0 and 3.0mg/l plasma CRP representing low, medium and high cardiovascular risk respectively (Ridker *et al.* 2002, 2005). Periodontitis is an example of a common disease that results in an elevated CRP concentration with plasma CRP significantly raised in patients with chronic periodontitis compared to healthy controls with a mean high-sensitivity CRP range of 1-4mg/l (Fredriksson *et al.* 1999, Loos *et al.* 2000, Paraskevas *et al.* 2008, Offenbacher *et al.* 2009, D'Aiuto *et al.* 2010, Allen *et al.* 2011) thereby falling within the American Heart Association medium cardiovascular risk range (Ridker *et al.* 2002, 2005). A recent systematic review and meta-analysis also highlights modest evidence that systemic CRP concentrations are subsequently lowered following successful periodontal treatment (Paraskevas *et al.* 2008).

### **1.7.2 Functions of C-reactive protein**

Systemic CRP concentrations increase rapidly in response to trauma, inflammation and infection and similarly decrease rapidly with resolution and therefore the functions of CRP are thought to be directly related to the innate immune response (Du Clos *et al.* 2000). C-reactive protein binds in a calcium-dependent manner (Potempa *et al.* 1981, Du Clos *et al.* 2000) to different ligands including phosphorylcholine which is found in a number of host and bacterial cells (Oliveira *et al.* 1980, Potempa *et al.* 1981, Du Clos *et al.* 2000, Agrawal *et al.* 2002, Black *et al.* 2003, Lee *et al.* 2002) and becomes accessible to CRP only in damaged and apoptotic cells. C-reactive protein is therefore important in effectively scavenging cellular debris and extracellular matrix components (Pepys *et al.* 1983, Szalai *et al.* 1999), nuclear antigens such as chromatin and histones (Robey *et al.* 1984, Du Clos *et al.* 1988), as well as activation of the classical complement pathway through direct interaction with C1q (Taylor & van den Berg 2007). There is also evidence that CRP can interact with the Ig receptors Fc $\gamma$ RI and Fc $\gamma$ RII thereby eliciting a response from phagocytic cells including neutrophils (Du Clos *et al.* 2000, Black *et al.* 2004).

### **1.7.3 Neutrophil interaction with C-reactive protein**

Neutrophils are regarded as one of the most likely targets of CRP because CRP deposition occurs mainly at sites of tissue damage *in-vivo* within inflammatory sites infiltrated by neutrophils (Kushner *et al.* 1961, 1963, James *et al.* 1981, Mortensen & Duskiwicz 1977, Du Clos *et al.* 1981, Buchta *et al.* 1988, Shephard *et al.* 1990). Binding of CRP to neutrophils has been shown to occur via specific CRP receptors related to the neutrophil Fc receptor (Muller *et al.* 1986, Buchta *et al.* 1987). More specifically, soluble, pentameric CRP has been shown to bind rapidly and specifically to all three classes of human Fc $\gamma$ R (Fc $\gamma$ RI, RII and RIII) in a calcium- and magnesium-depended manner (Dobrinich & Spagnuolo 1991, Lu *et al.* 2008, Bottazzi *et al.* 2010). However, in terms of CRP binding to neutrophils, which normally only express low/medium affinity receptors for IgG (Fc $\gamma$ RII and Fc $\gamma$ RIII), most studies have highlighted the role of Fc $\gamma$ RIIA (Heuertz *et al.* 2005).

Soluble, pentameric CRP has been shown to have multiple effects on neutrophils, including inhibiting chemotaxis (Wood 1951, Buchta *et al.* 1987, Kew *et al.* 1990, Heuertz *et al.* 1993), promoting phagocytosis (Kindmark 1971, Mold *et al.* 1981, 1982, Holzer *et al.* 1984,

Kilpatrick *et al.* 1985, Buchta *et al.* 1987) and reducing the stimulated respiratory burst (Buchta *et al.* 1987, Filep & Foldes-Filep 1989, Dobrinich & Spagnuolo 1991). Low concentrations (<5mg/l) of CRP have been suggested to potentiate activation of neutrophil superoxide generation following PMA or ConA stimulation, although >10mg/l CRP significantly inhibited superoxide generation measured by cytochrome c reduction (Buchta *et al.* 1987). Similarly, purified human CRP at elevated concentrations of >50mg/l inhibited the respiratory burst of neutrophils triggered by a variety of stimuli including PMA and fMLP (Buchta *et al.* 1987, 1988, Kew *et al.* 1990, Dobrinich *et al.* 1991, Shephard *et al.* 1992). Relatively few studies have investigated physiologically relevant concentrations of CRP, although 0.3mg/l CRP has been shown to inhibit neutrophil superoxide generation following stimulation with fMLP or PMA (Buchta *et al.* 1988). C-reactive protein (8mg/l) was also found to inhibit neutrophil degranulation and superoxide production, and also stabilized the neutrophil membrane against the lytic effect of lysophosphatidylcholine (Filep *et al.* 1989). In contrast, CRP within the concentration range of 10-100mg/l has been reported to have no significant effect on hydrogen peroxide generation by stimulated neutrophils (Timoshenko *et al.* 1998). To date there are no published studies investigating physiologically-relevant concentrations of CRP on the neutrophil respiratory burst in the presence of periodontally-relevant bacteria.

## **1.8 THESIS AIMS & OBJECTIVES**

The pathogenesis of chronic periodontitis remains to be fully elucidated. However, the majority of evidence suggests that a dysregulated neutrophil inflammatory-immune response results in collateral host tissue damage within the periodontal tissues (Chapple & Matthews 2007, Nussbaum & Shapira 2011; section 1.2.2). Therefore, the overall aim of this thesis was to contribute to our current understanding of the role of the neutrophil in the pathogenesis of chronic periodontitis along with possible associations with systemic disease (such as cardiovascular disease) in which raised systemic CRP concentrations are a risk factor.

In order to achieve these aims, the work described in this thesis had the following specific objectives:



**To establish and perform a case-controlled longitudinal intervention study providing neutrophils from periodontitis patients, pre- and post-therapy, and from matched periodontally healthy controls volunteers**

The first objective was to recruit patients diagnosed with chronic periodontitis along with age- and gender-matched periodontally- and systemically-healthy control volunteers. Clinical measures were performed to confirm the presence and absence of disease for patient and healthy control volunteers respectively. All volunteers provided baseline peripheral blood samples for neutrophil isolation and plasma preparation prior to patients progressing through conventional quadrant-by-quadrant periodontal treatment as clinically indicated. Post-therapy blood samples were collected for all patient volunteers and their paired controls. Clinical measures were performed on all patient volunteers post-therapy in order to determine treatment effectiveness and to support any observed differences in *ex vivo* neutrophil function.

**To determine neutrophil cytokine release in chronic periodontitis and the effect of non-surgical periodontal therapy**

IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  release from neutrophils isolated from patients with chronic periodontitis pre- and post-therapy and matched healthy controls was determined by ELISA analysis of supernatants from 18-hour cell cultures. Given the increasing awareness of the ability of neutrophils to secrete cytokines and co-ordinate the inflammatory-immune response this will determine whether aberrant cytokine release from peripheral blood neutrophils may be involved in the pathogenesis of chronic periodontitis.

**To determine neutrophil extracellular superoxide release in chronic periodontitis and the effect of non-surgical periodontal therapy**

Extracellular O<sub>2</sub><sup>-</sup> release from neutrophils isolated from patients with chronic periodontitis pre- and post-therapy and matched healthy controls was determined by lucigenin-enhanced chemiluminescence. Although over-production of ROS by neutrophils is a well-established feature of chronic periodontitis, investigating the specific role of O<sub>2</sub><sup>-</sup> will further elucidate the role of ROS in disease pathogenesis.

**To determine neutrophil extracellular trap release in chronic periodontitis**

Neutrophil extracellular trap release from patients with chronic periodontitis pre- and post-therapy and matched healthy controls was determined using a recently developed fluorometric assay. Data from these novel experiments will add to our current understanding of the pathogenesis of chronic periodontitis, especially in relation to the hyper-responsive neutrophil ROS phenotype, since ROS are essential for NET release.

**To determine neutrophil chemotactic accuracy in chronic periodontitis**

Neutrophil directional chemotactic accuracy from patients with chronic periodontitis pre- and post-therapy and matched healthy controls was determined, for the first time, using a novel direct visualisation chemotaxis chamber. The data obtained will be important because enhanced recruitment into, and/or delayed tissue transit times through, periodontal tissues by hyper-responsive neutrophils will have the potential to increase collateral host tissue damage.

**To determine the effect of CRP on the neutrophil respiratory burst**

The final objective of this thesis was to determine the effects of physiologically-relevant concentrations of CRP on the neutrophil respiratory burst *in vitro* using chemiluminescence methods. This will add to our current understanding of neutrophil function in relation to systemic inflammatory markers that are associated with both chronic periodontitis and systemic disease.

## **CHAPTER 2**

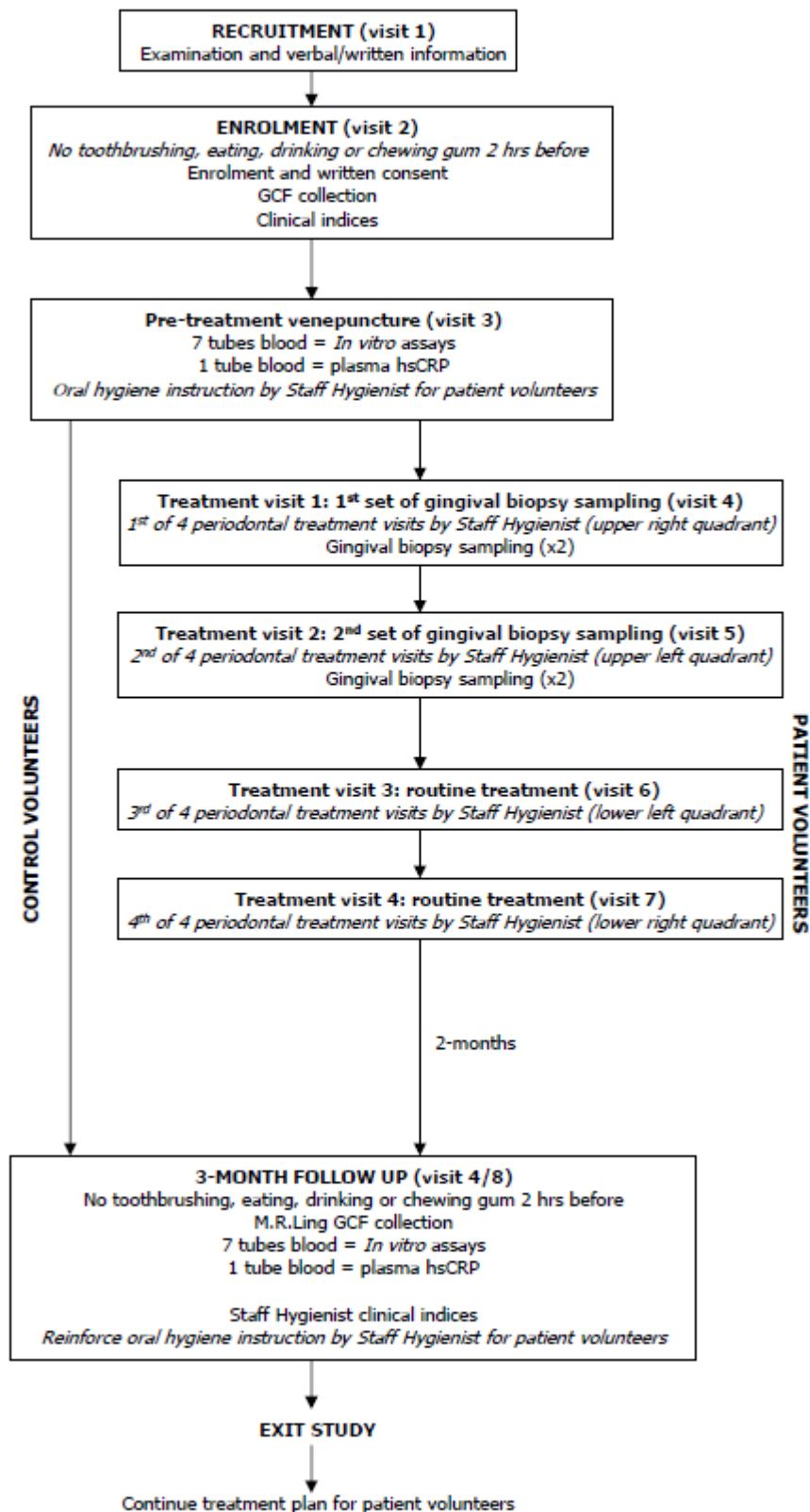
### **MATERIALS & METHODS**

## **2.1 CLINICAL METHODS**

All clinical procedures were performed by M.R.Ling including recruiting all patient and control volunteers, collection of clinical measurements, gingival crevicular fluid (GCF) and gingival biopsy sampling. Clinical support was provided by J.Crumpler (Research Nurse) regarding dental nursing, performing venipuncture for collection of peripheral blood samples and organising patient appointments. Additional clinical support was provided by J.Rooney (Research Hygienist) who performed periodontal treatment for all patient volunteers.

### **2.1.1 DETERMINATION OF NEUTROPHIL FUNCTION IN PERIODONTITIS PATIENTS PRE- & POST-NON-SURGICAL THERAPY**

This was performed using a longitudinal interventional case-control clinical study including patients diagnosed with chronic periodontitis and matched periodontally and systemically healthy control volunteers (Figure 2.1). Volunteers progressed through the study with sampling visits pre- and post-non-surgical periodontal therapy. Patient volunteers subsequently progressed through 4 visits of quadrant-by-quadrant periodontal treatment (section 2.1.1.6) on a weekly basis. Patients were then re-assessed 2-months following treatment of the 4<sup>th</sup> quadrant to allow for initial healing consistent with conventional periodontal treatment protocols and to reduce the risk of re-infection/disease re-activation (Chapple *et al.* 2007) impacting upon post-treatment measures. The review appointments included repeat clinical measures to assess response to treatment along with post-therapy sample collection. Healthy control volunteers were recalled and sampled at the same time as their matched patient volunteer. The sample size (20 patients and 20 controls; n=40) was based upon previous studies of neutrophil responses within the Periodontal Research Group in order to obtain a >80% power to detect significant differences between test and control volunteer neutrophils at  $p<0.05$  by two-tailed statistical analyses.



**Figure 2.1. Longitudinal interventional clinical study flow diagram.** Note the sampling visits for the patient and control volunteers.

#### **2.1.1.1 Ethical approval**

Ethical approval for the clinical study was sought using the online Integrated Research Application System (IRAS; reference no. 46998) and granted by the West Midlands Research Ethics Committee (REC reference no. 10/H1208/48). The study was subsequently adopted onto the National Institute of Health Research Network Portfolio (NIHR UKCRN Study ID 10318) and eligible for additional financial support from the UK NIHR Clinical Research Network. The study was also added to ClinicalTrials.gov (Identifier NCT01233765). Sponsorship was obtained from the University of Birmingham (Sponsor reference no. RG\_10-077) and NHS Permission was granted by the Birmingham Community Health Care NHS Trust on behalf of the Birmingham and the Black Country Comprehensive Local Research Network (BBC CLRN) Research Management & Governance (RM&G) Consortium Office (NHS R&D reference no. R&D1398; RM&G reference no. BCHCTDent265.1398.P). The study was classified as a clinical study and came under the Department of Health Research Governance Framework for Health and Social Care.

#### **2.1.1.2 Funding**

Funding for the clinical study was obtained from the Oral and Dental Research Trust (£6,273; RCFL14945), the British Society of Periodontology (£4,444; RCRW14944) and the Royal College of Surgeons of England (£9,200; RCFX16209). Additional funding was also obtained from BBC CLRN (£15,938) to cover the costs of a research nurse and hygienist.

#### **2.1.1.3 Recruitment**

Volunteers enrolled included those diagnosed with chronic periodontitis along with age- and gender-matched periodontally and systemically healthy controls. All volunteers were never-smokers and otherwise systemically healthy as confirmed by a detailed medical history questionnaire (appendix I). Chronic periodontitis was defined as the presence of at least two non-adjacent sites with probing pocket depths >4mm, along with radiographic bone loss  $\geq 30\%$  of the root length (non-first molar or incisor sites) in accordance with the consensus criteria of the European Federation of Periodontology (Tonetti & Claffey 2005). Control volunteers had no evidence of attachment loss, no probing pocket depths >4mm and whole-mouth bleeding scores <10%. Inclusion criteria included the complete absence of systemic

disease, pregnancy, special dietary needs, vitamin supplements and no anti-inflammatory or antibiotic medication in the previous 3 months. Age and gender-matched periodontally and systemically healthy control volunteers were recruited from Staff of the Birmingham Dental Hospital and School. Volunteers were initially provided with a study information leaflet (appendix II), participation discussed and questions answered prior to providing written informed consent. All volunteers were then re-appointed for clinical measures, sample collection and periodontal treatment as appropriate. Accrual recruitment data was uploaded on a monthly basis onto the UK Clinical Research Network Portal (<https://portal.ukcrn.org.uk>) as a condition of continued support from the BBC CLRN.

#### **2.1.1.4 Clinical measures**

The following clinical measures and samples (section 2.1.1.5) were collected from the patient and healthy control volunteers as indicated. Gingival crevicular fluid samples (section 2.1.1.5b) were collected initially, followed by the gingival index measurements (section 2.1.1.4a), periodontal probing pocket depths (section 2.1.1.4c), gingival recession (section 2.1.1.4d), and finally the plaque index measurements due to the additional use of plaque-disclosing solution (section 2.1.1.4b). Patient and healthy control volunteers were re-appointed for peripheral blood sampling (section 2.1.1.5a) immediately prior to periodontal treatment for the patient volunteers, during which time gingival biopsy samples were collected as indicated (section 2.1.1.5c).

##### ***a. Gingival index***

The presence of gingival inflammation was measured using the Löe (1967) gingival index (GI; Löe 1967). The teeth were dried using a triple air syringe, and a periodontal probe (Hu-Friedy Manufacturing Co., Chicago, IL) was gently introduced into the gingival crevice at a force of ~0.25N at 60° to the long axis of the tooth. 3 buccal/labial and 3 palatal/lingual thirds on each tooth (6 sites in total) were analysed according to standard criteria (Table 2.1).

##### ***b. Plaque index***

The presence of dental plaque was measured using the Quigley & Hein, Turesky & Lobene modified (1982) plaque index (PI; Quigley & Hein 1962, Turesky *et al.* 1970, Lobene *et al.* 1982). Plaque was visualized by gently brushing the surface of the teeth with a cotton bud

soaked in plaque-disclosing solution. A visual assessment of the presence of plaque was subsequently undertaken on 3 buccal/labial and 3 palatal/lingual thirds on each tooth (6 sites in total) according to standard criteria (Table 2.2).

***c. Periodontal probing pocket depths***

Full mouth periodontal probing pocket depths (PPD) were measured using a PCP-UNC-15 periodontal probe (Hu-Friedy Manufacturing Co., Chicago, IL) with a force of ~0.25N along the long axis of each tooth. PPD was measured as the distance between the gingival margin and the base of the periodontal pocket/sulcus at a total of 6 sites per tooth, with 3 measurements on each of the buccal/labial and palatal/lingual surfaces.

***d. Gingival recession***

Full mouth gingival recession measures were performed using a PCP-UNC-15 periodontal probe (section 2.1.1.4c), and measured as the distance between the cement-enamel junction and the gingival margin at a total of 6 sites per tooth, with 3 measurements each on the buccal/labial and palatal/lingual surfaces.

***e. Clinical attachment loss***

Clinical attachment loss (CAL) was calculated as the sum of the PPD and gingival recession measurements in accordance with clinical and histological studies of periodontal attachment loss (Haffajee & Socransky 1986).

***f. Bleeding on probing***

Bleeding upon periodontal probing was recorded in conjunction with the PPD measurements (section 2.1.1.4c) indicating bleeding from the base of the periodontal pocket, as well as during probing at 60° to the long axis of the teeth during the measurement of GI (section 2.1.1.4a), thereby distinguishing from bleeding from the surrounding sulcular/pocket epithelium.

***g. Classification of chronic periodontitis***

Chronic periodontitis was classified as localized (<30% sites) or generalized (≥30% sites) and severity determined, namely mild (1-2mm), moderate (3-4mm) or severe (>4mm) based on CAL calculations (Armitage 1999, Milward & Chapple 2003; section 2.1.1.4e).



Gingival index	Condition	Criteria		
		Gingival colour	Gingival margin	Gingival tissue
0	Health	Pale pink or pink	At or apical to the amelo-cemental junction	Firm to palpation with a blunt probe
1	Mild inflammation	Red or bluish-red tinge	Colourless exudate may be present. No bleeding on periodontal probing	Little change in texture of any portion of, but not the entire marginal or papillary unit
2	Mild to moderate inflammation	Red or reddish-blue glazed appearance	Enlarged and oedematous. No bleeding on periodontal probing	Change in texture involving the entire marginal or papillary gingival unit
3	Moderate to severe inflammation	Markedly red or bluish-red appearance	Oedema and/or hypertrophy, with bleeding on periodontal probing	Oedema and/or hypertrophy of the entire marginal or papillary gingival unit

**Table 2.1. Loe (1967) gingival index criteria.**

Plaque index	Criteria
0	Zero plaque
1	A discontinuous line of plaque at the gingival margin
2	A continuous line of plaque at the gingival margin up to 1mm in depth
3	A continuous line of plaque at the gingival margin >1mm extending up to 1/3 <sup>rd</sup> of the tooth surface
4	A continuous line of plaque at the gingival margin extending >1/3 <sup>rd</sup> up to 2/3 <sup>rd</sup> of the tooth surface
5	A continuous line of plaque at the gingival margin extending >2/3 <sup>rd</sup> of the tooth surface

**Table 2.2. Quigley & Hein, Lobene modification (1982) plaque index criteria.**

#### **2.1.1.5 Sample collection**

Clinical measurements (section 2.1.1.4), peripheral blood and gingival crevicular fluid (GCF) samples were collected for all patient and matched healthy control volunteers both pre- and post-periodontal treatment. In addition, patient volunteers progressed through 4 visits of quadrant-by-quadrant periodontal treatment (section 2.1.1.6) on a weekly basis which also included gingival biopsy harvesting.

##### ***a. Peripheral blood samples***

Peripheral blood was collected from the antecubital fossa into Vacutainers (Greiner, Bio-One Ltd., Stonehouse, UK; 6ml) containing lithium heparin (17IU/ml) as an anticoagulant. Blood was collected between 09:00 and 10:00am. Preliminary experiments confirmed a typical cell retrieval of  $1 \times 10^6$  neutrophils per ml of venous blood, and therefore a total of 7 tubes were required to have sufficient cells for the enhanced chemiluminescence assay of reactive oxygen species (ROS; section 2.2.8), fluorometric neutrophil extracellular trap analysis (section 2.2.9), directional chemotaxis (section 2.2.10) assays, as well as neutrophil culture prior to determination of cytokine release (section 2.2.6). One additional tube of blood was collected for plasma preparation (section 2.2.1), therefore equating to a total of 8 tubes of blood in total (~48ml) per volunteer.

##### ***b. Gingival crevicular fluid***

Gingival crevicular fluid (GCF) was collected prior to the clinical measures (section 2.1.1.4) using a Periopaper (OraFlow, Plainview, New York, NY, USA) inserted into the mesio-buccal gingival crevice of 6 teeth (a molar, pre-molar and canine or incisor) within the upper left and right sextants. Intra-oral sites were carefully isolated using cotton wool rolls placed within the buccal sulcus along with use of a saliva ejector. Air from a triple air syringe was gently directed towards the coronal aspect of the tooth to be sampled a total of 5 times from the gingivae to the occlusal surface away from the gingival crevice and not into the crevice. A Periopaper was removed from the holder using tweezers to grasp the orange section only, and the paper portion of the strip was gently inserted into the gingival crevice so that its leading edge was parallel to the line of the gingival margin, trying not to bend the strip. The Periopaper was left undisturbed for 30s prior to removal and read on a pre-calibrated Periotron 8000 (Chapple *et al.* 1999; OraFlow, Plainview, New York, NY, USA) for

calculation of GCF volume. 3 strips from each sextant were immediately placed into 400µl of 100mM ammonium bicarbonate buffer and stored at -80°C, prior to analysis of protein citrullination by mass spectrometry (section 2.2.11.3).

### ***c. Gingival biopsy samples***

During periodontal treatment patient volunteers received local anaesthesia prior to instrumentation according to standard treatment protocols. During the first two treatment visits, whilst anaesthetized, two 2mm diameter gingival biopsy samples were collected submarginally from an inflamed and adjacent non-inflamed gingival site, using a 2mm diameter biopsy punch (Schuco 33-31-P/25) and sharp dissection with a microsurgical blade, immediately prior to root surface debridement of the sampled sextant. Gingival biopsy samples were immediately placed into a Universal bottle containing 10% neutral buffered formalin (4% formaldehyde v/v) for 18-24hr fixation prior to routine processing to wax at the Human Biomaterials Resource Centre, the University of Birmingham. Gingival biopsy samples were collected in order to provide tissue for future determination of *in vivo* NET formation by immunohistochemical methods.

### **2.1.1.6 Periodontal treatment for patient volunteers**

Patient volunteers received tailored oral hygiene instruction and conventional non-surgical periodontal therapy. This consisted of scaling and root surface debridement (RSD) of all periodontal pockets >4mm performed under local anaesthesia on a quadrant-by-quadrant basis within a maximum of 4 weeks.

## **2.1.2 DETERMINATION OF THE EFFECT OF C-REACTIVE PROTEIN ON NEUTROPHIL FUNCTION**

Periodontally and systemically healthy volunteers were recruited and each donated a peripheral blood sample (section 2.1.1.5a) for isolation of peripheral blood neutrophils prior to determination of neutrophil reactive oxygen species (ROS) generation and release in the presence of C-reactive protein (CRP; section 2.2.11.8).

### **2.1.2.1 Ethical approval**

Ethical approval was issued by the University Ethical Review Committee (University of Birmingham Ethics Reference ERN\_10-0518), and Sponsorship provided by the University of Birmingham (Sponsor Reference RG\_10-122). The CRP experiments were classified as a Supervised Student Clinical Trial, for which M.R.Ling was Chief Investigator, and came under the Department of Health Research Governance Framework for Health and Social Care.

### **2.1.2.2 Volunteer selection**

Volunteers were recruited from staff and students of the Birmingham Dental Hospital and School. Inclusion criteria included volunteers to be systemically and periodontal healthy as determined by a detailed medical history questionnaire and periodontal examination, no pregnancy and no special dietary needs along with a complete absence of vitamin supplements and no use of anti-inflammatory or antibiotic medication in the previous 3 months. All volunteers were non-smokers for  $\geq 5$  yrs. Following identification, study participation was discussed and questions answered prior to obtaining written informed consent. Volunteers were then re-appointed for collection of a peripheral blood sample (section 2.1.2.3).

### **2.1.2.3 Peripheral blood sample**

Peripheral blood was collected from the ante-cubital fossa into Vacutainers (Greiner, Bio-One Ltd., Stonehouse, UK; 6ml) containing lithium heparin (17IU/ml) between 9:00 and 10:00hrs. According to the anticipated cell retrieval, 3 tubes of blood were required in order to ensure an adequate number of cells to undertake all planned enhanced chemiluminescence assay experiments (section 2.2.11.8).

## **2.2 LABORATORY METHODS**

All laboratory procedures were performed by M.R.Ling apart from analytical ultracentrifugation (section 2.2.11.4), mass spectrometry (section 2.2.11.3) and determination of plasma hsCRP concentrations (section 2.2.2). Analysis of neutrophil extracellular trap (NET) production (section 2.2.9) and neutrophil chemotaxis (section 2.2.10) were performed as part of collaborative projects with P.Harris and H.Roberts respectively who assisted with these analyses (see Acknowledgements).

### **2.2.1 Plasma preparation**

Blood in lithium-heparin Vacutainers<sup>TM</sup> (Greiner, Bio-One Ltd, Stonehouse, UK; 6ml) was centrifuged in sealed buckets at 1,000rcf for 30mins at 4°C (IEC Centra 3R). Post-centrifugation, Vacutainers<sup>TM</sup> were opened and the separated plasma carefully removed using a sterile plastic Pasteur pipette and aliquoted into cryogenic vials (0.5ml/tube) for storage at -80°C.

### **2.2.2 High-sensitivity C-reactive protein analyses of stored plasma samples**

High-sensitivity CRP (hsCRP) analyses were performed on 0.5ml aliquots of the stored plasma samples. This was undertaken by Dr Jonathan Aldis, UK National Protein Reference Centre, Immunology Department & Protein Reference Unit, Sheffield Teaching Hospitals NHS Foundation Trust.

### **2.2.3 Isolation of peripheral blood neutrophils**

Peripheral blood neutrophils were isolated from peripheral blood using the following reagents, prior to cell counting and confirmation of neutrophil purity and viability.

#### **2.2.3.1 Erythrocyte lysis buffer**

Lysis buffer was prepared by dissolving 8.3g NH<sub>4</sub>Cl (A9434 Sigma), 1g KHCO<sub>3</sub> (P9144 Sigma), 0.04g Na<sub>2</sub> EDTA 2H<sub>2</sub>O (E5134 Sigma) and 2.5g bovine serum albumin (BSA; A4530 Sigma) in 1L of sterile distilled water and stored at 4°C until use for a maximum of 2 weeks.

The cell density of granulocytes (1.08-1.09g/ml; Morisaki *et al.* 1992) is similar to that of erythrocytes (1.09-1.10g/ml; Linderkamp *et al.* 1993). An erythrocyte lysis step was therefore required during neutrophil isolation to remove any contaminating erythrocytes due to the pseudoperoxidase activity of haemoglobin (Thomas 1985, Widmer *et al.* 2010) along with light absorbing capacity when using chemiluminescence methods (Faber *et al.* 2003).

#### 2.2.3.2 Phosphate buffered saline (PBS)

PBS was prepared by dissolving 7.75g NaCl (S9625 Sigma), 0.2g KH<sub>2</sub>PO<sub>4</sub> (P5379 Sigma) and 1.5g K<sub>2</sub>HPO<sub>4</sub> (P8281 Sigma) in 1L of sterile distilled water. The solution was autoclaved and stored at 4°C until use for a maximum of 2 weeks.

#### 2.2.3.3 Percoll density gradients

Percoll is a silica colloid that is widely used as a density gradient medium to isolate cells from peripheral blood with high viability and intact morphology (Dooley *et al.* 1982). Percoll is comprised of a silica sol with a non-dialyzable polyvinylpyrrolidone coating, and a density of 1.130±0.005g/ml. Percoll was chosen rather than alternative neutrophil separation solutions as many of the experiments involved myeloperoxidase (MPO) catalyzed formation of hypochlorous acid. Ficoll-Hypaque density gradients were therefore avoided since it is possible that I<sup>-</sup> may leach from the diatrizoate and compete with Cl<sup>-</sup> for oxidation by the MPO-H<sub>2</sub>O<sub>2</sub>-halide system (Grisham 1985). In addition, Ficoll isolation techniques are also thought to pre-activate neutrophils (Rebecchi *et al.* 2000).

Neutrophils were isolated using Percoll densities of 1.098g/ml and 1.079g/ml (Bergstrom & Asman 1993), prepared using sterile reagents according to the equation:

$$V_o = V \frac{\rho - 0.1\rho_{10} - 0.9}{\rho_o - 1}$$

**Where:**

V<sub>o</sub> = volume of Percoll (undiluted) (ml)

V = volume of the final working solution (ml)

ρ = desired density of the final solution (g/ml)

$\rho_0$  = density of Percoll (undiluted) (g/ml) (exact density specified in the “Certificate of Analysis”)

$\rho_{10}$  = density of 1.5M NaCl = 1.058 (g/ml)

The following volumes (ml) were used to prepare Percoll solutions:

	Percoll Density (g/ml)	
	1.079	1.098
<b>Percoll</b>	19.708	24.823
<b>dH<sub>2</sub>O</b>	11.792	6.677
<b>1.5M NaCl</b>	3.5	3.5
<b>Total volume (ml)</b>	<b>35</b>	<b>35</b>

To prepare discontinuous density gradients, 8ml of 1.098g/ml Percoll was carefully layered underneath 8ml 1.079g/ml Percoll in a sterile 25ml centrifuge tube using a sterile plastic Pasteur pipette. Gradients were prepared immediately prior to neutrophil isolation.

#### 2.2.3.4 Neutrophil isolation

Peripheral blood was carefully layered onto discontinuous Percoll density gradients ( $\delta=1.079:1.098$ ) within a sterile 25ml centrifuge tube and then centrifuged (IEC Centra CL3R) for 8mins at 150rcf, followed by 10mins at 1,200rcf (Figure 2.2).

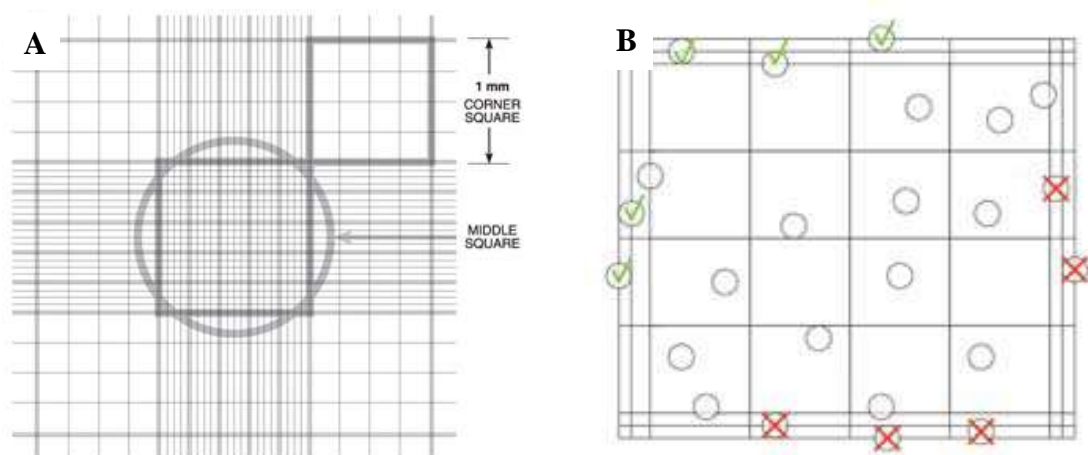


**Figure 2.2. Peripheral blood separation using Percoll density gradient centrifugation.** The neutrophil layer may be observed immediately above the separated erythrocytes at the bottom of the centrifuge tube.

Using a sterile plastic Pasteur pipette, the plasma, lymphocyte and monocyte layers at the top of the centrifuge tube above the neutrophil and erythrocyte cell layers, were carefully removed and discarded. The neutrophil layer, located at the top of the erythrocyte cell layer, was then carefully transferred to a 50ml centrifuge tube containing ~20ml erythrocyte lysis buffer and the final volume made up to ~50ml with additional erythrocyte lysis buffer. The tube was gently inverted to mix, and incubated at room temperature for 5-10mins until the erythrocytes had lysed and the cell suspension changed from cloudy to transparent. The lysed cell suspension was then centrifuged for 6mins at 500rcf to pellet the neutrophils. The supernatant was carefully discarded using a sterile plastic Pasteur pipette and the pellet re-suspended in an additional 3ml lysis buffer and incubated for 5mins at room temperature prior to re-centrifugation (6mins; 500rcf). The supernatant was once again removed and the pellet washed in 3ml PBS, and centrifuged once more for 6mins at 500rcf prior to re-suspension in 4-8ml PBS depending on the size of the cell pellet prior to cell counting (section 2.2.3.5).

### 2.2.3.5 Neutrophil cell counting

Neutrophils were counted using a haemocytometer (Sigma Z359629) whose central area was  $1\text{mm}^2$ , subdivided into 25 ( $0.04\text{mm}^2$ ) smaller squares surrounded by triple lines, each subdivided further into 16 smaller squares as shown (Figure 2.3).



**Figure 2.3. Haemocytometer central viewing area (A) &  $0.04\text{mm}^2$  squares surrounded by triple lines (B) with example cell count (Sigma Z359629 product information sheet).**

Cells were counted if they were within the triple lines either above or to the left-hand side of the  $0.04\text{mm}^2$  squares, but not the right-hand side or bottom to ensure a uniform cell count



(Figure 2.2). The depth of the haemocytometer was 0.1mm, and therefore the number of cells within the PBS suspension was calculated according to the equation:

$$\frac{\text{Cell count} \times 250 \times 1,000}{9} = \text{no. cells in 1ml cell suspension}$$

9

**Where:**

Cell count = the number of cells counted within a total of nine 0.04mm<sup>2</sup> squares within the diagonal axis of the central 1mm<sup>2</sup> area of the haemocytometer.

Following counting, the required amount of buffer (PBS, GPBS or RPMI) was added to the cell preparation to give a final cell suspension containing the appropriate neutrophil concentration required for the different assays. These included 1x10<sup>6</sup>/ml for the neutrophil ROS enhanced chemiluminescence assays (section 2.2.8.2) and the NET fluorometric assays (section 2.2.9.2); 3.125x10<sup>6</sup>/ml for neutrophil cell culture (section 2.2.6) and 2x10<sup>6</sup>/ml for the directional chemotaxis assay (section 2.2.10.1).

#### **2.2.3.6 Confirmation of neutrophil purity and viability**

Isolated cells were >99% polymorphonuclear cells morphologically and devoid of erythrocytes and monocytes. The absence of monocytes in cells prepared using this method has also been confirmed previously within our laboratory by gene expression analysis (Wright *et al.* 2008). Giemsa stained cell smears (Barcia 2007) also confirmed the presence of >99% neutrophils in the cell preparation.

Neutrophil cell viability was routinely determined by trypan blue dye exclusion (1:1 mix of cells with trypan blue; T8154 Sigma) and visualization by light microscopy (Leitz Laborlux). Neutrophil viability was expressed as the percentage of cells not permeable to trypan blue. In addition, for some experiments, cell metabolic activity was also analysed using a CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay Kit (Promega, Southampton, UK) according to the manufacturer's instructions. Briefly 100µl of CellTiter-Glo<sup>®</sup> Reagent was added to 100µl of neutrophil cell suspension in a 96-well plate and incubated for 10mins prior to recording luminescence using a microplate reader (Bertold microplate luminometer LB96v). This

method is based upon quantifying adenosine triphosphate (ATP) which signals the presence of metabolically active cells.

#### **2.2.4 Preparation of bacterial stimuli**

All bacteria were originally obtained commercially and stored in tryptone soya broth supplemented with 10% DMSO at -80°C in 2ml cryovials. Initial inoculae were established by defrosting the frozen aliquots at room temperature prior to pouring onto tryptone soya agar plates and anaerobic incubation for 7 days with plates facing upwards. Bacteria were then streak plated onto fresh tryptone soya agar plates and incubated for a further 7 days to observe individual colonies indicating a pure culture (Holt 1994). Specific culture conditions for each individual bacterial species are described below.

##### **2.2.4.1 Tryptone soya broth**

Tryptone soya broth (CM0129 Oxoid) was prepared according to the manufacturer's instructions, namely 9g tryptone soya broth was dissolved in 300ml distilled water. 10ml of the tryptone soya broth was aliquotted into borosilicate glass culture tubes (FB59537 Fisher Scientific) with vented plastic caps (TB51373 Fisher Scientific) and autoclaved at 121°C for 15mins prior to storage at 4°C.

##### **2.2.4.2 Enriched tryptone soya broth**

Enriched tryptone soya broth, required for the growth of *Porphyromonas gingivalis* (ATCC 33277), was prepared by addition of 1mg/ml glucose powder and 0.01ml per ml hemin to the standard tryptone soya broth (section 2.2.4.1) prior to autoclaving and 0.3µg/ml vitamin K added after autoclaving.

##### ***a. Hemin***

50mg hemin (Sigma H-5533) and potassium phosphate 1.74g (Sigma P-5504) were added to 100ml of distilled water. This solution was boiled until the hemin had dissolved. After cooling to room temperature, the solution was stored at 4°C covered in aluminium foil for a maximum of three weeks.

#### ***b. Vitamin K***

5mg menadione (Sigma M-5625) was dissolved in 1ml of 95% ethanol (Sigma). 99ml distilled water was added and the resultant solution filter sterilized (0.2µm; Corning 430944) prior to storage at -20°C for a maximum of three weeks.

#### **2.2.4.3 Tryptone soya agar**

Tryptone soya agar (CM0131 Oxoid) was prepared by dissolving 40g in 1L distilled water according to the manufacturer's instructions. After autoclaving at 121°C for 15mins the agar was cooled to 50°C in a water bath before 5% defibrinated horse blood (SR0050B Oxoid) was added aseptically. Agar was poured into triple vented Petri dishes (101VR20 Sterilin) and left inverted for 24hrs to allow ventilation of excess moisture, prior to storage at 4°C. Before use, dishes were allowed to equilibrate at room temperature for 1hr.

#### **2.2.4.4 Enriched tryptone soya agar**

The growth of *Porphyromonas gingivalis* required the use of agar enriched with hemin (section 2.2.4.2a), N-acetylmuramic acid (NAM; section 2.2.4.4a) and vitamin K (section 2.2.4.2b). Ten hemin-NAM-vitamin K (HNK) agar plates were prepared by adding 5.0g tryptone soya agar (section 2.2.4.3), 6.5g brain heart infusion (CM225 Oxoid), 2.5g yeast extract (Y-1001 Sigma) and 2.5ml hemin (section 2.2.4.2a) to distilled water (final volume = 250ml). This solution was sterilized by autoclaving at 121°C for 15mins and then cooled to 50°C before adding 300µl NAM (section 2.2.4.4.a), 2.5ml vitamin K (section 2.2.4.2b) and 12.5ml defibrinated horse blood (SR0050B Oxoid) before pouring into 90mm triple vented Petri dishes and being allowed to set at room temperature.

#### ***a. N-acetylmuramic acid***

100mg of N-acetylmuramic acid (NAM; Sigma A3007) was dissolved in 10ml of distilled water and filter sterilized (0.2µm, BC591 Appleton Woods) prior to storage at -20°C for a maximum of three weeks.

#### **2.2.4.5 Determination of bacterial growth by spectrophotometry**

Post-culture bacterial proliferation was determined by suspending the bacterial cell pellets in sterile PBS prior to measuring optical density at 600nm (OD<sub>600nm</sub>) using a spectrophotometer

(Jenway 6300), with samples of non-inoculated media used to correct for media changes not due to bacterial growth. *Porphyromonas gingivalis* (ATCC 33277) and *F. nucleatum* (ATCC 10953) OD<sup>1.0</sup><sub>600nm</sub> were 1.69x10<sup>9</sup> and 1.62x10<sup>9</sup> bacteria/ml respectively as pre-determined by the Forsyth Institute, Boston, USA (Roberts *et al.* 2002).

#### **2.2.4.6 *Porphyromonas gingivalis***

*Porphyromonas gingivalis* is a Gram negative anaerobe that is part of the red complex of bacteria considered central to the pathogenesis of chronic periodontitis (Socransky *et al.* 1998), and interacts with neutrophil Toll-like receptors (TLR)-2 and -4 (Darveau *et al.* 2004).

*Porphyromonas gingivalis* (ATCC 33277) was obtained from the Forsyth Institute, Boston and was originally purchased from the American Type Culture Collection (ATCC). *Porphyromonas gingivalis* was grown anaerobically at 37°C as previously described (Roberts *et al.* 2002) within an anaerobic chamber (Don Whitley Scientific, Modular Atmosphere Controlled System, CAL-3200, Shipley, UK). The atmosphere comprised of 9.97% carbon dioxide, 9.92% hydrogen, with nitrogen balance at 37°C. *Porphyromonas gingivalis* cultures were maintained on enriched tryptone soya agar and isolated by centrifugation at 700rcf for 10mins and subsequently washed 3 times in sterile PBS before being heat-treated at 100°C for 10mins prior to dilution in sterile PBS giving a final suspension of 4.12x10<sup>9</sup> cells/ml (section 2.2.4.5) which was stored at -30°C.

#### **2.2.4.7 *Fusobacterium nucleatum***

*Fusobacterium nucleatum* is a gram-negative anaerobe present at high frequency and number in the subgingival plaque biofilm in periodontal lesions and is central to periodontal disease pathogenesis as the microflora develops from a non-pathogenic to a pathogenic state (Kolenbrander *et al.* 1995; Socransky *et al.* 1998). *F. nucleatum* is also a key quorum sensing organism present in subgingival plaque (Kolenbrander *et al.* 1995). Heat-killed *F. nucleatum* activates neutrophils via TLR-2, -4 and -9, but predominantly via TLR-2 (Kikkert *et al.* 2007).

*Fusobacterium nucleatum* (ATCC 10953) was obtained from the Forsyth Institute, Boston and originally purchased from the American Type Culture Collection. *Fusobacterium*

*nucleatum* was grown anaerobically at 37°C as previously described (Roberts *et al.* 2002) within an anaerobic chamber as described for *P. gingivalis*. *F. nucleatum* cultures were maintained on broth cultures and isolated by centrifugation at 700rcf for 10mins and subsequently washed 3 times in sterile PBS before being heat-treated at 80°C for 10mins prior to dilution in sterile PBS giving a final stock suspension of  $8.5 \times 10^9$  cells/ml (section 2.2.4.5) which was stored at -30°C.

#### **2.2.4.8 *Staphylococcus aureus***

*Staphylococcus aureus* (National Collection of Type Cultures; NCTC 6571, Heatley 1944) was obtained from Fisher Scientific and stored at -40°C in cryo-preserved on beads (Prolab PL160). One bead was retrieved and used to inoculate a mannitol salt agar plate for growth of single colonies indicated by a yellow colouration of agar. Following an aerobic overnight incubation (5% CO<sub>2</sub>, 37°C), several colonies were used to inoculate 500ml sterile tryptone soya broth in a glass flask, using a flame sterilized loop. The inoculated broth was incubated overnight (5% CO<sub>2</sub>, 37°C), following which the culture contained stationary phase bacteria (approx.  $10^9$ /ml) as indicated with the broth turning opaque. Bacterial numbers were also confirmed using colony counts by streak plating and also measuring optical density before and after washing. The culture was divided into 10 separate 50ml centrifuge tubes (Fisher Scientific), and bacteria pelleted by centrifugation for 10mins at 700rcf (IEC Centra CL3R). The supernatant was discarded into a waste bottle for disposal by autoclaving and the pellets were re-suspended in 20ml PBS by vortex mixing. Suspensions in PBS were pooled into 4 separate 50ml tubes, re-centrifuged, washed again by re-suspension in 20ml PBS, pooled into two tubes and then washed a third time by re-suspension in 20ml PBS. All harvesting and washing procedures were undertaken in a Biological Safety cabinet (AirOne 1000-GS, Safelab Systems Ltd) using aseptic technique.

Following the third re-suspension and washing step, the pellets were then each re-suspended in 20ml 3% formaldehyde (P6148 Sigma) for 1 hour at room temperature to fix. The fixative was removed by centrifugation and the cells washed a further three times by re-suspension in 20ml PBS (20ml for each pellet). Finally the pellets were re-suspended in 5ml PBS at a concentration of  $1 \times 10^{11}$  bacteria/ml and stored at -80°C.

#### **a. Opsonisation of *Staphylococcus aureus***

*Staphylococcus aureus* was opsonized with IgG to specifically stimulate neutrophil Fc-gamma receptors (FcγR). Opsonised *S. aureus* was prepared as previously described (Bergstrom *et al.* 1993) by the addition of 33μl Vigam liquid (5mg/ml IgG; Bio Products Laboratory) per ml of bacteria and incubated overnight at room temperature with constant agitation. Finally bacteria were re-suspended in PBS and stored as a  $3.75 \times 10^9$  cells/ml suspension in 1ml aliquots at -80°C prior to use.

#### **2.2.4.9 *Escherichia coli* O26:B6 lipopolysaccharide**

Lipopolysaccharide is a component of the outer membrane of Gram-negative bacteria and acts primarily via TLR-4 ligation (Hoshino *et al.* 1999). *E. coli* LPS (Sigma L5543) was supplied as a 0.2μm filtered aqueous solution containing 1mg/ml of lipopolysaccharide (LPS) from *E. coli* serotype O26:B6. The LPS used to prepare the solution was phenol-extracted and chromatographically purified by gel filtration as confirmed by the product information sheet

Neutrophil responses to LPS are dependent upon binding to membrane CD14 (Nakatomi *et al.* 1998) in the presence of plasma or LPS binding protein (Kirkland *et al.* 1993, Luchi *et al.* 1993). Lipopolysaccharide samples were therefore diluted in 20% human plasma (Sigma H4522) and PBS using siliconised pipette tips (Cole-Parmer 25711-32, Cole-Parmer 25711-56) and siliconised microcentrifuge tubes (Sigma T4816), and stored at -20°C prior to use.

#### **2.2.5 Preparation of non-bacterial stimuli**

Non-bacterial stimuli included heat-aggregated IgG for neutrophil FcγR stimulation and phorbol 12-myristate, 13-acetate (PMA) which bypasses cell surface receptors and directly activates protein kinase C (PKC; Johnson *et al.* 2002). In addition hypochlorous acid (HOCl) was included as a recently established stimulus for neutrophil extracellular trap (NET) formation (Palmer *et al.* 2012).

##### **2.2.5.1 Heat-aggregated IgG**

Freeze-dried IgG purified from human serum (Sigma I4506) was re-constituted in 150mM NaCl to give a 4.8mg/ml solution as determined by spectrophotometry ( $E_{280}^{1\%} = 14$ ). Subsequent dilutions in PBS were heated in a glass tube in a water bath at 63°C for 60mins

(Zeller *et al.* 1986) and allowed to cool to room temperature prior to use. Heat-aggregated IgG was prepared immediately prior to use. The formation of aggregated IgG was confirmed using analytical ultracentrifugation analyses (AUC; section 2.2.11.4).

#### **2.2.5.2 Phorbol 12-myristate, 13-acetate**

Phorbol 12-myristate, 13-acetate (PMA; Sigma P8139) was reconstituted with DMSO to give a 1mg/ml (1.62mM) stock solution that was stored at -20°C prior to use. Working solutions were prepared by diluting this stock solution with PBS.

#### **2.2.5.3 Hypochlorous acid**

Hypochlorous acid (HOCl) was prepared by diluting sodium hypochlorite in sterile water. The concentration of hypochlorite ions was estimated by optical density at 292nm of pH 12.0 solutions using molar absorptivity with a 350M/cm extinction coefficient (Morris 1966). The final pH, when used in experiments, was approximately the same as the pKa for HOCl (7.5), and it was therefore assumed that 50% existed as HOCl and 50% existed as OCl<sup>-</sup> (Palmer *et al.* 2012).

#### **2.2.6 Neutrophil culture**

Neutrophils were cultured primarily to determine their ability to release cytokines within an 18 hour culture period. However, the cell pellets from the cultures were also retained for future gene expression analyses.

Freshly isolated neutrophils in PBS were centrifuged at 500rcf for 6mins prior to re-suspension at a concentration of  $3.125 \times 10^6$ /ml in RPMI-1640 (Sigma R8758) supplemented with 0.3g/l glutamine, 2.32g/l HEPES, 2g/l sodium bicarbonate, 100µg/ml streptomycin and 100UI/ml penicillin (1% of 10mg/ml streptomycin, 10,000U/ml penicillin; Sigma P4333) and 10% heat-inactivated FBS. 400µl of cell suspension ( $1.25 \times 10^6$  neutrophils) was added to each well within a 24-well cell culture plate (NUNC 142465, Thermo, Fisher Scientific UK TKT-190-010Y). Thereafter, 100µl of stimulant diluted in supplemented RPMI (5µg/ml LPS final well volume, *F. nucleatum* and *P. gingivalis* at MOI 100:1 and opsonized *S. aureus* at MOI 300:1) or additional supplemented RPMI was added to each well and the cells incubated for

18h at 37°C and 5% CO<sub>2</sub>. Bacterial MOI's were chosen in relation to previous studies investigating neutrophil responses using a similar culture technique (Hatanaka *et al.* 2006) including that of the enhanced chemiluminescent assay to measure neutrophil reactive oxygen species release (section 2.2.8.2; Matthews *et al.* 2007) . Furthermore, an MOI of 100:1 is the same as used to study the effects of periodontal bacteria on epithelial cell function and comparable to estimates of the maximum number of bacteria associated with epithelial cells within periodontal pockets (Dierickx *et al.* 2002; Milward *et al.* 2013).

After culture, the neutrophil cell suspensions were carefully collected individually using a sterile plastic Pasteur pipette, a sample of which was used to determine post-culture neutrophil viability by trypan blue dye exclusion (section 2.2.3.6). Individual cell suspensions were then centrifuged in a sterile microtube at 1,800rcf for 10 minutes (Spectra merlin) and the supernatant carefully removed, taking care not to disturb the cell pellet, and transferred to a separate labeled sterile microtube and initially stored at -80°C in 2ml aliquots.

### **2.2.7 Enzyme-linked immunosorbent assays**

Enzyme-linked immunosorbent assays (ELISAs) were used to assay supernatants from cultured neutrophils for the presence of IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$ . Commercial ELISA kits (R&D Systems, Abingdon, UK) were used for all assays which were performed according to the kit manufacturer's instructions. Preliminary ELISA experiments were performed to determine sample dilution and standard ranges within the quoted assay sensitivities. All reagents and samples were allowed to equilibrate to room temperature before use, and all standards, samples and controls were assayed in duplicate.

#### **2.2.7.1 General ELISA reagents & procedures**

The following reagents and procedures were used for the ELISA assays as stated.

##### ***a. Wash buffer and well washing***

ELISA wash buffer was prepared by diluting 20ml wash buffer concentrate (895003; R&D Systems) into 480ml distilled water. Although not clear in the kit instructions, R&D Systems confirmed that the wash buffer was the same for all four ELISA kits employed. During washing stages, each well was aspirated and washed with 400 $\mu$ l wash buffer three times for a



total of four washes using a plate autowasher (BioTek ELx50). After the last wash, the plate was inverted and blotted against clean paper towels in order to remove any excess wash buffer.

***b. Assay diluents***

Assay diluents (buffered protein base; R&D Systems) were added to the IL-6 (895117; 100µl), IL-8 (895117; 100µl) and TNFα (895041; 50µl) ELISA plates immediately prior to addition of the standards and samples.

***c. Conjugate antibodies***

Following incubation of the standards and samples, and well washing (section 2.2.7.1a), 200µl of polyclonal antibody conjugated to horseradish peroxidase against IL-6 (890046; R&D Systems), IL-1β (890040; R&D Systems), TNF-α (892539; R&D Systems) or alternatively 100µl IL-8 conjugate (890465; R&D Systems) was added per well prior to covering with a new adhesive strip and incubation for 2h for IL-6, or alternatively 1h for IL-8, IL-1β and TNF-α.

***d. Substrate and stop solutions***

Colour reagents A (stabilized hydrogen peroxide; 895000; R&D Systems) and B (stabilized chromogen; tetramethylbenzidine; 895001; R&D Systems) were mixed together in equal volumes within 15mins of use and protected from light. At the end of the ELISA procedure, 200µl of the substrate mixture was added to each well. Following incubation (20mins for IL-6, IL-1β and TNF-α and 30mins for IL-8) the reaction was stopped by addition of stop solution (2N sulphuric acid; 895032; R&D Systems). At this point the colour of the solutions in the wells changed from blue to yellow. If the color of the solution in the wells was green or the colour change was not uniform then the microplate was gently tapped in order to ensure thorough mixing.

***e. Determination of optical density***

The optical density (450nm and 570nm) of each well was determined in duplicate as soon as possible following addition of the ELISA stop solution (section 2.2.7.1d) using a microplate reader (BioTek ELx800) set to 450nm. Additional readings were collected at 570nm which

were later subtracted from the readings at 450nm in order to correct for optical imperfections in the plate (ELISA product information sheet, R&D Systems).

***f. Calculation of ELISA cytokine sample concentrations***

The mean of duplicate readings ( $OD_{450nm}$  minus  $OD_{570nm}$ ) for each standard and sample were calculated, and the mean zero standard (blank) optical density subtracted. Individual standard curves were then plotted using the mean absorbance for each standard on the x-axis against the concentration on the y-axis and a 3<sup>rd</sup> order polynomial regression trend line added through the points on the graph using Microsoft Excel (Microsoft, Washington, USA; Figure 2.4). The sample concentrations were calculated from the equation describing the 3<sup>rd</sup> order polynomial regression trend line and then multiplied by the individual dilution factors. Where sample ODs were outside the pre-determined standard ranges, repeat analyses were performed at an adjusted sample dilution. Outliers were calculated as those >1.5x interquartile range (1.5xIQR; Tukey 1977), and tabulated where present.

***g. ELISA sensitivities and minimal detectable doses***

Sample recovery and minimal detectable doses were obtained from the respective product information sheets supplied from R&D Systems for each of the cytokines investigated, namely IL-6 (98%; <0.70pg/ml), IL-8 (98%; ~3.5(1.5-7.5)pg/ml), IL-1 $\beta$  (97%; <1pg/ml) and TNF- $\alpha$  (98%; ~1.6(0.5-5.5)pg/ml).

**2.2.7.2 Basic ELISA protocol**

Quantikine ELISA kits (R&D Systems, Abingdon, UK) were used for the quantitative determination of human IL-6 (S6050; LOT no. 310797), IL-8 (S8000C; LOT no. 309261), IL-1 $\beta$  (SLB50; LOT no. 311937) and TNF- $\alpha$  (STA00C; LOT no. 310802) within the culture supernatants.

Individual sample dilutions and standard ranges were pre-determined using additional samples prior to analysis of the main study samples as outlined in Table 2.3, with standards prepared by serial dilution in the respective calibrator diluent for IL-6 (RD5T; 895175), IL-8 (RD5P; 895151), IL-1 $\beta$  (RD5-5; 895485) and TNF- $\alpha$  (RD6-35; 895360).

IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  ELISAs were performed according to the relevant product instructions as summarized in Table 2.4.

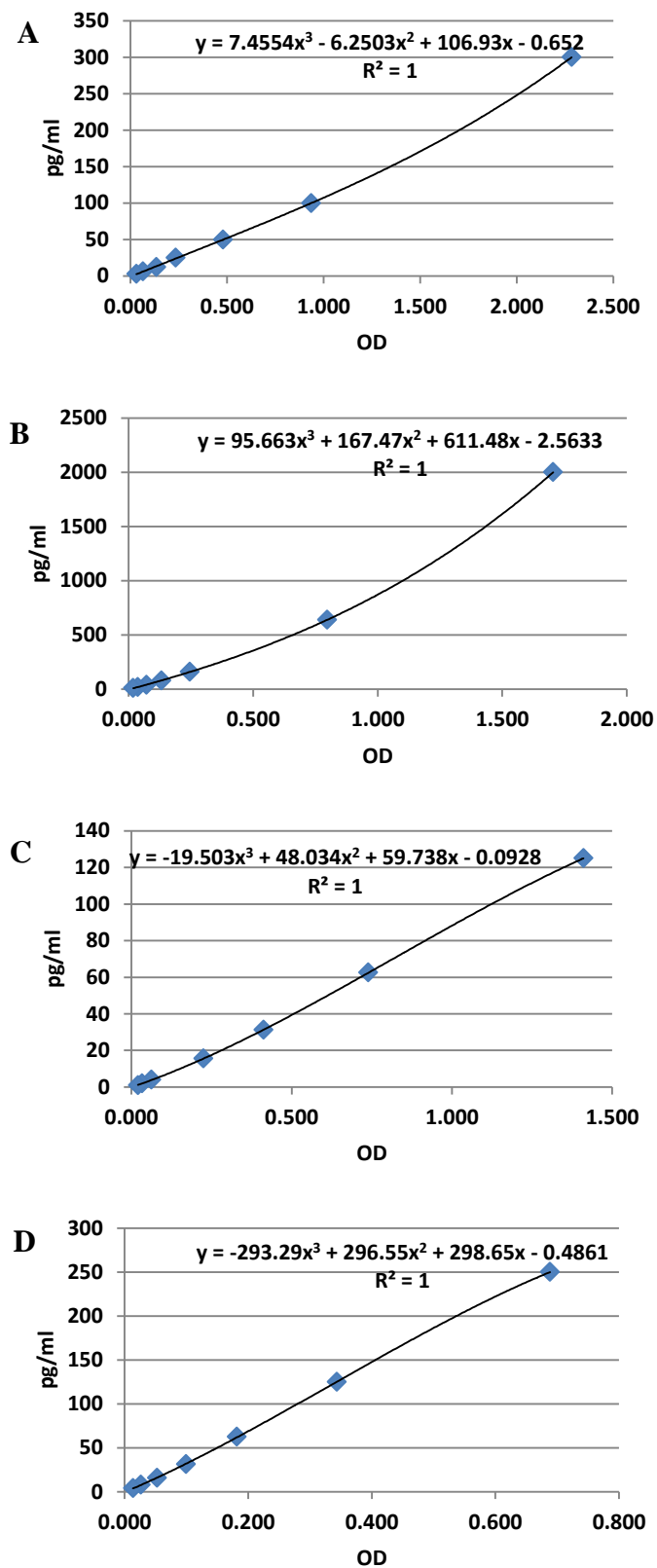


Figure 2.4. Typical standard curves for IL-6 (A), IL-8 (B), IL-1 $\beta$  (C) and TNF- $\alpha$  (D).

Cytokine	Sample dilution	Standard ranges (pg/ml)							
		0	3.12	6.25	12.5	25	50	100	300
<b>IL-6</b>	1:3	0	3.12	6.25	12.5	25	50	100	300
<b>IL-8</b>	1:40	0	10	20	40	80	160	640	2,000
<b>IL-1<math>\beta</math></b>	Neat	0	1	2	3.9	15.6	31.2	62.5	125
<b>TNF-<math>\alpha</math></b>	Neat	0	3.9	7.8	15.6	31.2	62.5	125	250

**Table 2.3. Sample dilutions and standard ranges used in the IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  ELISAs (R&D Systems, Abingdon, UK).**

Reagent	Cytokine			
	IL-6	IL-8	IL-1 $\beta$	TNF- $\alpha$
<b>Assay diluent</b>	100 $\mu$ l	100 $\mu$ l	None	50 $\mu$ l
<b>Standard/sample</b>	100 $\mu$ l	50 $\mu$ l	200 $\mu$ l	200 $\mu$ l
<b>Incubation at room temperature</b>	2h	2h	2h	2h
Plate washing (section 2.2.7.1a)				
<b>Conjugate</b>	200 $\mu$ l	100 $\mu$ l	200 $\mu$ l	200 $\mu$ l
<b>Incubation at room temperature</b>	2h	1h	1h	1h
Plate washing (section 2.2.7.1a)				
200 $\mu$ l substrate solution (section 2.2.7.1d)				
<b>Incubation at room temperature (protected from light)</b>	20mins	30mins	20mins	20mins
50 $\mu$ l stop solution (section 2.2.7.1d)				

**Table 2.4. Overview of IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  ELISA procedures according to the individual kit instructions (R&D Systems, Abingdon, UK).**

### 2.2.8 Chemiluminescence methods for detection of neutrophil derived ROS

There are different techniques that may be used to measure cellular release of ROS, largely involving photometry, fluorometry and luminometry (reviewed by Dahlgren & Karlsson 1999). These include peroxidase-dependent (luminol and isoluminol) and lucigenin-amplified chemiluminescence, advantages of which include high sensitivity and the ability to follow the kinetics of the response. In addition, luminol, isoluminol and lucigenin-enhanced chemiluminescence techniques are well established within our laboratory, with data directly comparable to published values (Matthews *et al.* 2007, 2011, 2012, Wright *et al.* 2008, 2011, Dias *et al.* 2011, Palmer *et al.* 2012, Chapple *et al.* 2013, Ling *et al.* 2014).

Chemiluminescent substrates are excited by oxygen radicals and emit light when returning to the ground state (Allen & Loose 1976). Luminol and isoluminol amplified chemiluminescence requires the presence of a peroxidase, such as myeloperoxidase (MPO) originating from azurophilic neutrophil granules. Luminol is able to cross the cell membrane predominantly detecting the intracellular MPO-catalyzed formation of hypochlorous acid derived from hydrogen peroxide (Dahlgren *et al.* 1983, Lundqvist *et al.* 1994). Isoluminol differs from luminol in only one respect, namely the position of the amino group in the phthalate ring of the molecule. This difference makes it more hydrophilic and membrane-impermeable, whilst not affecting the light generating reaction (Dahlgren & Karlsson 1999). Thus isoluminol measures extracellular reactive oxygen species in the presence of peroxidase, if present. In practice, in the laboratory, horse radish peroxidase (HRP) is added to the assay mixture in order to compensate for a relative lack of secreted neutrophil extracellular MPO (Lundqvist 1995, 1996). The isoluminol assay therefore predominantly detects extracellular HRP-catalyzed formation of hypochlorous acid (HOCl) derived from extracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). In contrast, lucigenin is a larger molecule (511Da) than luminol or isoluminol (177Da) and amplifies the chemiluminescent response by an MPO-independent mechanism directly related to the presence of superoxide (O<sub>2</sub><sup>-</sup>; Williams *et al.* 1981, Hasegawa 1997). Lucigenin is also impermeable to cells and therefore measures specifically extracellular O<sub>2</sub><sup>-</sup> release (Dahlgren *et al.* 1985).

### **2.2.8.1 Buffers, chemiluminescent reagents and multiwell plates/strips**

All reagents for the enhanced chemiluminescence assays were added to the multiwell plates following plate washing and immediately prior to the addition of cells and subsequent plate-reading. All reagents were equilibrated to room temperature prior to use, with all chemiluminescent substrate tubes and multiwell plates covered with tin foil and out of natural and artificial light where possible in order to prevent reagent degradation as stated in the relevant product information sheets.

#### ***a. Blocking buffer***

10g bovine serum albumin (BSA; A4503 Sigma) was dissolved in 1L PBS to give 1% BSA in PBS. The solution was aliquoted into working volumes of ~20ml and stored at -20°C for a maximum of four weeks.

#### ***b. Glucose-supplemented PBS***

Previous studies have demonstrated that both glucose and cations are required for optimal superoxide production by neutrophils (Tan *et al.* 1998). Whilst glucose alone can transiently activate neutrophils, its presence at 1mM has been shown to have no effect on neutrophil metabolism and oxidant release (Kummer *et al.* 2007).

Glucose-supplemented PBS (GPBS) was produced by adding 1.8g glucose (1mM), 0.15g CaCl<sub>2</sub> (1.5mM; 10070 BDH) and 1.5ml MgCl<sub>2</sub> (1M; 22093 BDH) to 1L of PBS. Each reagent was added and mixed completely prior to the addition of the next reagent in order to ensure thorough dissolution of all added reagents. Supplemented PBS was stored at 4°C for a maximum of 4 weeks.

#### ***c. Luminol & isoluminol***

30mM stock solutions of luminol (3-Aminophthalhydrazide, 5-Amino-2,3-dihydro-1,4-phthalazinedione; A8511 Sigma) and isoluminol (6-Amino-2,3-dihydro-1,4-phthalazinedione; A8264 Sigma) were prepared by dissolving 0.5g luminol/isoluminol in 94.05ml 0.1M NaOH. These stock solutions were tin foil-wrapped and stored at 4°C for a maximum of one week. The 3mM working solutions were prepared by diluting 1ml stock luminol or isoluminol with 9ml PBS, pH checked and adjusted where necessary to pH 7.3. The final well concentrations

for use in the ROS assays were 0.45mmol/l for luminol and 0.9mmol/l for isoluminol respectively (Bergstrom & Asman 1993, Matthews *et al.* 2007).

***d. Horseradish peroxidase***

A 1,000U/ml stock solution of horseradish peroxidase (Sigma P8415) in PBS was prepared and stored at -20°C. The working solution (100U/ml) was prepared by diluting the stock 1:10 with PBS, with 15µl added to a 200µl final well volume resulting in 1.5U/well in the ROS assays.

***e. Lucigenin***

A 1mg/ml stock solution of lucigenin (N,N'-Dimethyl-9,9'-biacridium dinitrate; Sigma M8010) in PBS was prepared and stored at 4°C for a maximum of 1 week. A 0.33mg/ml working solution was prepared by diluting the stock 1:3 with PBS, with 30µl added to the 200µl ROS assay wells resulting in a 0.05mg/ml final well concentration (Blair *et al.* 1988).

***f. Multiwell plates/strips***

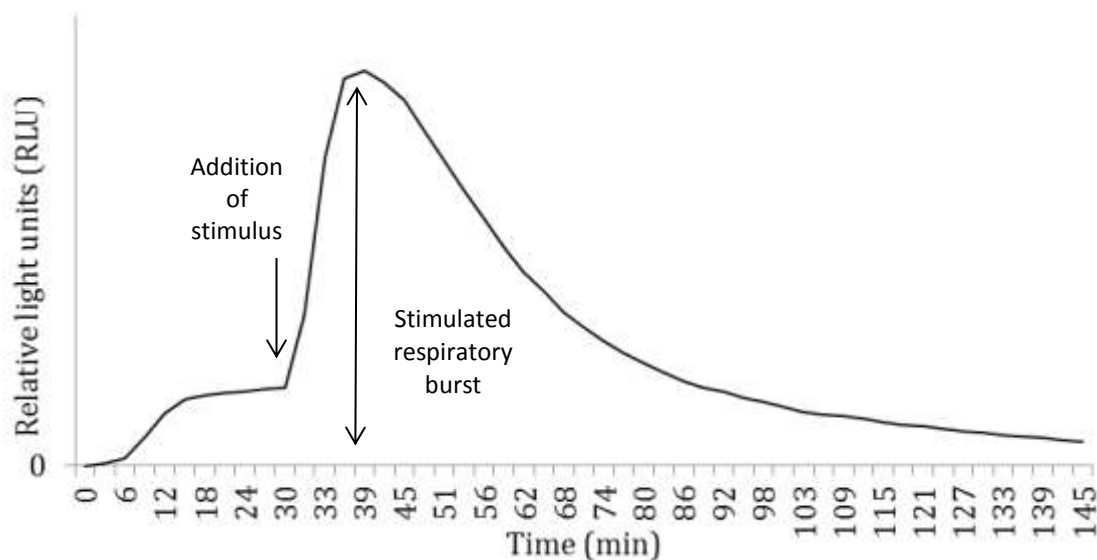
96-well white plates (Microlite2; 7567 Thermo Scientific, Dynex) were used for all enhanced chemiluminescence assays. Plates were blocked (PBS BSA 1%, overnight at 4°C) and washed with PBS using an automated plate washer (BioTek ELx50) immediately prior to addition of reagents and cells.

**2.2.8.2 Enhanced chemiluminescence assay**

30µl GPBS was added to pre-blocked white 96-well plates (2.2.8.1f). To detect predominantly intracellular hypochlorous acid (HOCl) generation, 30µl luminol (3mmol/l; pH 7.3; section 2.2.8.1c) was added. Alternatively, either 60µl isoluminol (3mmol/l; pH 7.3; section 2.2.8.1c) and 1.5U horseradish peroxidase diluted in PBS (HRP; Sigma P8415; 15µl; section 2.2.8.1d) were added to detect predominantly extracellular HOCl, or, to specifically detect extracellular superoxide release, 30µl lucigenin (0.33mg/ml; section 2.2.8.1e) was added. Following the addition of reagents, 100µl of neutrophil suspension (1x10<sup>6</sup> cells/ml in GPBS; 1x10<sup>5</sup> cells) was added and the plate immediately transferred to the plate reader and light output recorded for 1s per well in relative light units (Berthold microplate luminometer LB96v Berthold Technologies UK Ltd) at 37°C. Baseline light output was recorded for 30mins before addition



of stimulating agent or vehicle control (25µl), giving a total volume of 200µl per well. Light output was then recorded for a further 120 minutes (Figure 2.5).



**Figure 2.5. Schematic representation of the neutrophil respiratory burst using enhanced chemiluminescent techniques.** Please note baseline readings prior to the addition of a stimulus after 30mins resulting in increased light output representative of the stimulated respiratory burst.

All analyses were controlled within the same plate, since day-to-day variation in luminol chemiluminescence is thought to be  $17.4 \pm 8.8\%$  (Bergstrom & Asman 1993). All samples were analysed in quadruplicate, with paired patient and control cells isolated and analysed at the same time. Peak height was used for assessment of chemiluminescence kinetics, as this is considered a reliable parameter (Allen 1986) and corresponds with the area under the curve (Allen 1986, Hasegawa 1997).

## 2.2.9 Determination of neutrophil extracellular trap (NET) production

Given that DNA is a major structural component of NETs (Brinkmann et al 2004), the fluorescent DNA stain Sytox Green (Invitrogen S7020) was used to visualize and quantify NET release from peripheral blood neutrophils (Palmer *et al.* 2012).

### 2.2.9.1 NET visualization using Sytox Green

Neutrophils ( $1 \times 10^5$ ; suspended in 300µl RPMI) were seeded onto 1% PBS BSA (overnight, 4°C; 2.2.8.1a) coated 24-well plates (Microlite2; 7567 Thermo Scientific, Dynex) and allowed

to settle for 30 minutes in a 5% CO<sub>2</sub> atmosphere at 37°C. The neutrophils were then stimulated with 100µl PMA (25nM final concentration) or HOCl (0.75mM final concentration) for 3h at 37°C, 5% CO<sub>2</sub>. Following incubation, 25µl of 10µM Sytox Green (1:500; Invitrogen S7020; 1µM final concentration) was added. NETs were subsequently visualized at room temperature using a fluorescent microscope (Nikon Eclipse TE300, Kingston upon Thames, UK) with fluorescent filter excitation 450-490nm and emission 520nm (BrightLine® GFP-3035B, Semrock) at x20 magnification. Images were obtained using a digital camera (Nikon D5000).

#### **2.2.9.2 Fluorometric assay**

Fluorometric assays of NET release were performed using black 96-well microplates (3915 Corning) that had been pre-blocked with 200µl syringe-filtered 1% PBS BSA overnight at 4°C. 75µl media (RPMI Sigma R7509 without phenol red, supplemented with 0.5% L-glutamine), were added per well along with 1x10<sup>5</sup> neutrophils in 100µl RPMI (1x10<sup>6</sup>/ml). After a 30 minute baseline incubation period (37°C, 5% CO<sub>2</sub>), selected wells were stimulated with 25µl of PMA (Sigma P8139; final well concentration 25nM; Brinkmann *et al* 2004) or HOCl (final well concentration 0.75mM; Palmer *et al.* 2012) and incubated for 3h at 37°C, 5% CO<sub>2</sub>. Following incubation, 15µl of 14.3U/ml micrococcal nuclease (MNase; LS004797; Worthington Biochemical Corporation LS004797; final well concentration 1U/ml) was added per well and incubated at room temperature for 10 minutes prior to plate centrifugation (Hettich Universal 320R, 10mins at 1,800rcf) in order to pellet the cells and debris. Following centrifugation, 150µl of the supernatant was transferred to a separate sterile black 96-well plate (3915 Corning, Appleton Woods), taking care not to disturb the cell pellet. Extracellular NET-DNA, free within the supernatant, was quantified fluorometrically by adding 15µl of 10µM Sytox Green (Invitrogen S7020; final well concentration 0.9mM). The fluorescence from each well was recorded ten times at 37°C in arbitrary fluorescence units (AFU) using a fluorometer (Twinkle LB970, Berthold Technologies, Harpenden, UK) with an excitation wavelength of 485nm and an emission wavelength of 525nm.

#### **2.2.10 Determination of neutrophil directional chemotaxis (chemotactic accuracy)**

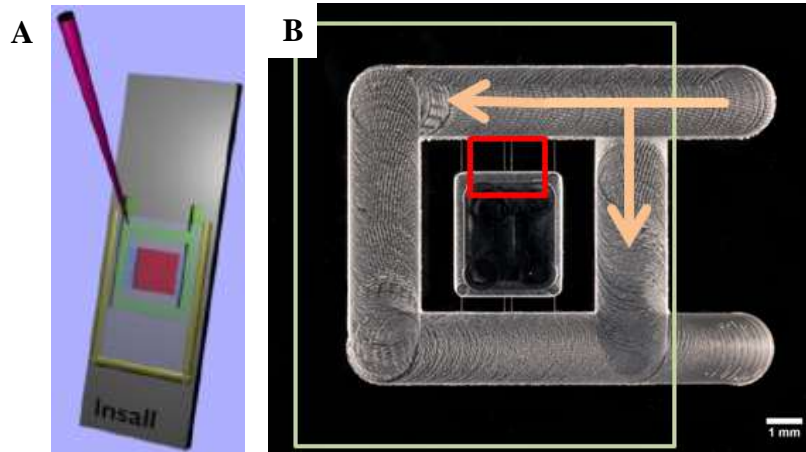
The majority of studies investigating neutrophil chemotaxis measure end points of cell movement using trans-well methods such as Boyden chambers. These methods essentially

gain indirect quantitative data by counting cells that have migrated into the chemoattractant well within a fixed time, and whilst these methods are useful they effectively only measure unidirectional movement of cells, more representative of chemokinesis, which is the random migration of cells observed in a homogenous solution of an extracellular signal (Wells 2000). In contrast, direct visualization chambers allow individual cell migration to be observed using time-lapse microscopy more representative of chemotaxis. A novel direct visualization chemotaxis (“Insall”) chamber has recently been developed by colleagues at the University of Glasgow (Muinonen-Martin *et al.* 2010) and has subsequently been used to measure neutrophil chemotaxis in patients diagnosed with chronic obstructive pulmonary disease (COPD; Sapey *et al.* 2011). Similar experiments have not yet been performed with regards to neutrophil chemotaxis in chronic periodontitis patients.

#### **2.2.10.1 Directional chemotaxis assay**

In order to inhibit non-specific adhesion, dry, acid-washed coverslips (22mm<sup>2</sup>, #1.5, 0.16-0.18mm; 0.2M HCl, 15 min followed by distilled water; 15 min) were coated in 400µl 7.5% serum albumin fraction whilst placed on filter paper. Excess serum albumin solution was removed and 400µl of a 2x10<sup>6</sup>/ml neutrophil cell suspension in RPMI supplemented with 1% BSA (Sigma A4812) were then placed on the coverslip and incubated for 30 minutes at room temperature. At the end of the incubation period, the Insall chemotaxis chamber (Muinonen-Martin *et al.* 2010; Figure 2.6) was washed three times with 400µl RPMI and a droplet of RPMI immediately placed on the chamber surface. The coverslip was then gently inverted and carefully lowered onto the chemotaxis chamber/RPMI droplet, resting one edge of the coverslip before gently lowering to ensure the central chamber remained bubble free and that the pipette loading bays remained exposed. Excess RPMI surrounding the coverslip and within the chemoattractant well was then carefully removed using filter paper and taking care not to displace the cover slip. 80µl IL-8 (10nM; R&D Systems, Abingdon, Oxfordshire, UK), formyl-methionyl-leucyl-phenylalanine (fMLP; 100nM; Sigma-Aldrich, Dorset, UK) or RPMI control was then injected into the chemoattractant well using a microloading tip. Chemoattractant concentrations were determined in agreement with pilot data collected whilst validating the experimental aspects of this assay within our laboratory and in accordance with the initial publication outlining this technique with patients diagnosed with chronic obstructive pulmonary disease (Sapey *et al.* 2011). The chemotaxis chamber was then viewed

using a Zeiss Primovert microscope (Carl Zeiss Ltd, Herts, UK) at x40 magnification, and images captured using a Q Imaging Retiga 2000R camera. All chemotaxis experiments were performed within a measured temperature range of 18.2-23.2°C in an air-conditioned laboratory.

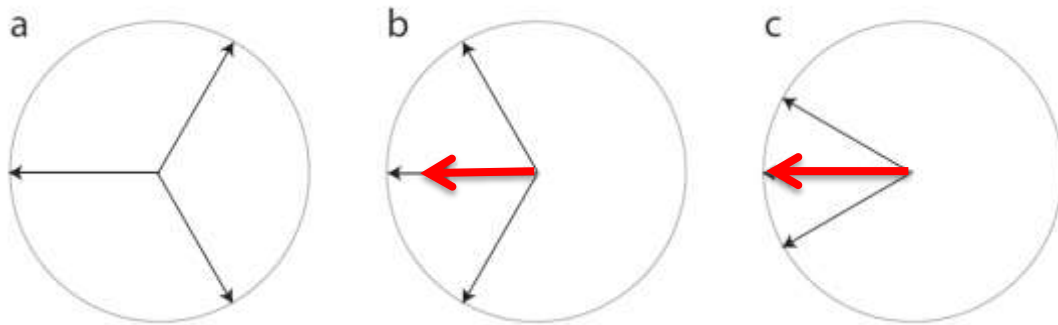


**Figure 2.6. Directional chemotaxis (Insall) chamber (Muinonen-Martin *et al.* 2010).** Please note the positions of the wells in relation to the chamber along with the position of the micropipette tip with which to introduce the chemoattractant (A). A detailed view of the wells displaying the outline of the inverted coverslip in relation to the chemoattractant wells, along with the viewing area highlighted in red (B).

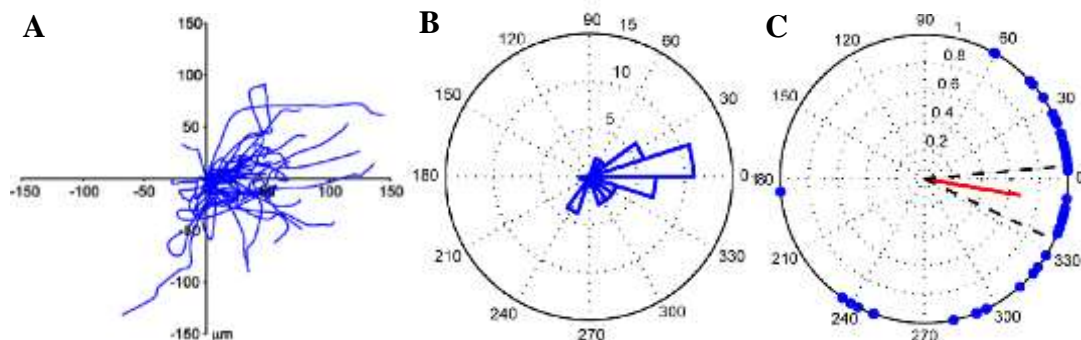
### 2.2.10.2 Time-lapse videomicroscopy image analysis

Images were generated every 30 seconds for 20 minutes resulting in 40 image stacks per experiment. Images were processed using Q proimaging software (Q Imaging Canada). Fifteen cells were chosen at random as those considered representative of the whole image area from the first image frame (Sapey *et al.* 2011). Chosen cells were subsequently tracked through the 40 image stacks using java software Image J 1.45SR software (Wayne Rasband; National Institutes of Health, Bethesda, Maryland, USA) with a manual tracking plug-in (MtrackJ) to determine XY coordinates of individual cells. The numerical data generated was then exported into Microsoft Excel (Microsoft, Washington, USA) prior to determination of chemotactic index, cell velocity and speed using pre-determined criteria and equations (Section 2.2.10.3; Sapey *et al.* 2011) and spider diagrams. The circular statistics (CircStat) toolbox from MATLAB (MathWorks Inc, Massachusetts, USA) was used to produce the angular histograms (rose plots) and resultant vector plots. The CircStat toolbox provided statistics for directional data including calculation of the mean resultant vector length using pre-determined equations, the length and orientation of which indicates the direction and magnitude of the mean cell migration (Figure 2.7; Berens 2009). The original spider diagram,

angular histogram and rose plots obtained using this novel technique are shown as an example (Figure 2.8; Muinonen-Martin *et al.* 2010).



**Figure 2.7. Example illustrations of the calculation of mean resultant vector lengths** (Berens 2009). Three equally separated migratory cells (A) yield a resultant vector length of zero since the points are uniformly spaced around the circle. Three migratory cells at 120, 180 and 240° (B) will result in a mean resultant vector length of  $2/3$  (0.67) towards 180°. Three migratory cells at 150, 180 and 210° (C) will yield a mean resultant vector length of  $5/6$  (0.83) towards 180°.



**Figure 2.8. Example spider diagrams (A), angular histograms (rose plots) (B) and resultant vector plots (C) representative of the migration of 43 MV3 human melanoma cells over 12 hours as published to initially describe the improved chamber for direct visualisation of chemotaxis** (Muinonen-Martin *et al.* 2010). In these experiments the chemoattractant (foetal bovine serum) was to the right of the images. The spider diagram (A) represents individual cell migration calibrated to the zero starting point with the angular histogram (B) representing cell migration predominantly towards the source of the chemoattractant. The small circles on the circumference of the resultant vector plot (C) representative of the end-points of each individual cell movement, with the central red line displaying the mean resultant vector length highlighting the direction and magnitude of the mean cell migration bounded by the 95% confidence intervals.

### **2.2.10.3 Calculation of chemotactic index, speed and velocity of neutrophil migration**

Chemotactic index, speed and velocity of neutrophil migration were calculated as previously reported (Sapey *et al.* 2011). Chemotactic index (a measure of the accuracy of neutrophil directional orientation) was calculated from the individual frame-by-frame cosines of the angles between the cell's migratory direction and the orientation of the chemoattractant gradient. Neutrophil speed of movement was calculated by measuring the distance travelled between frames and representative of random movement in any direction (chemokinesis). Velocity was also calculated as neutrophil speed of migration in a consistent direction towards the chemoattractant.

### **2.2.11 C-reactive protein**

C-reactive protein (CRP) from human plasma (2mg/ml) was obtained commercially (Sigma C4063) and supplied as a solution in 20mM Tris, pH 7.8-8.2, containing 280mM sodium chloride, 5mM calcium chloride and 0.1% sodium azide as a preservative. Prior to examining the effects of CRP on neutrophil ROS production, removal of sodium azide was required as this is known to inhibit MPO, HRP and catalase activity (Ortiz de Montellano *et al.* 1988, Van Uffelen *et al.* 1998) that are directly involved in both neutrophil ROS generation as well as the *ex vivo* ROS assay methodology.

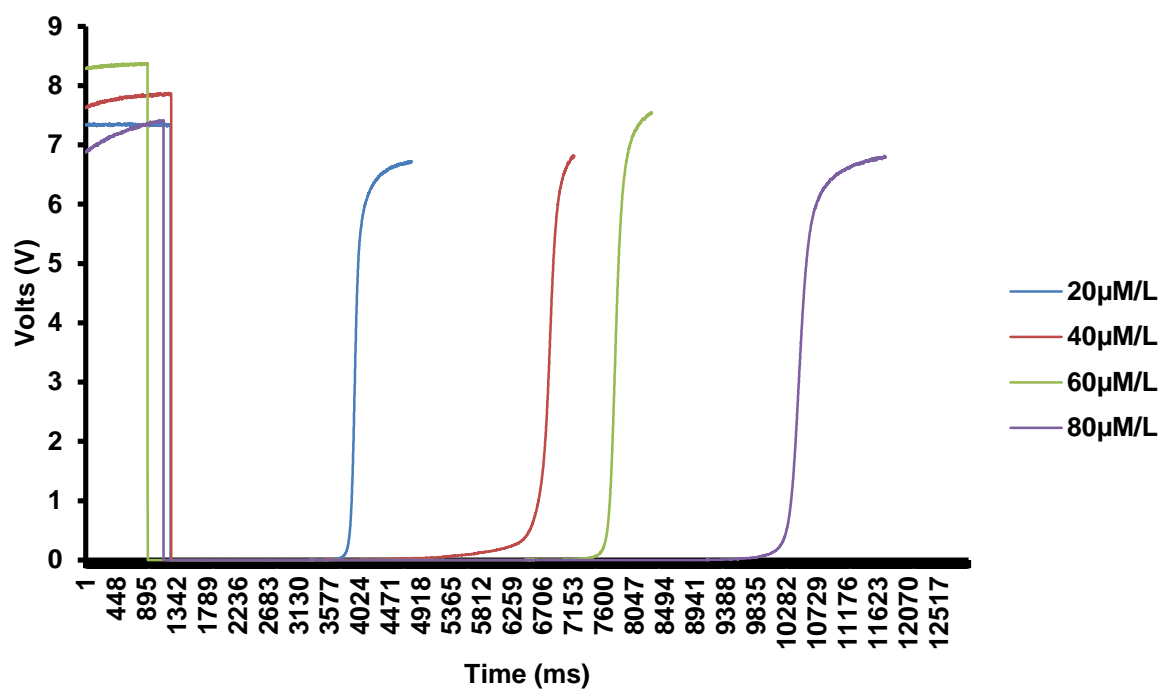
#### **2.2.11.1 Determination of the antioxidant capacity of CRP**

The luminol-based enhanced chemiluminescence antioxidant assay measures the antioxidant capacity of biological fluids (Brock *et al.* 2004, Chapple *et al.* 2007). Reactive oxygen species are generated that subsequently oxidise luminol and emitting a flash of light. By incorporating an enhancer species (*p*-iodophenol) a prolonged glow of light results from radical re-cycling, which is inhibited by antioxidant scavengers when added to the reaction (Chapple *et al.* 1997). HRP was used to initiate the reaction and the resulting light was measured using a tube luminometer (BioOrbit 1250 luminometer, Labtech International, UK) and a PICO data logger 200/12 analogue digital converter and accompanying software (Pico Technology Ltd). The antioxidant assay was calibrated on the day of sample analysis using a water-soluble vitamin-E analogue (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; Trolox). Standard curves were constructed using a series of Trolox standards (20, 40, 60 and 80µM in PBS containing 50mg/L BSA) run in triplicate immediately before and upon completion of the assays of CRP samples. 100µl of signal reagent (from a pre-equilibrated stock solution; Amerlite<sup>TM</sup>, Johnson & Johnson Clinical Diagnostic Ltd; Amersham UK) were added to a

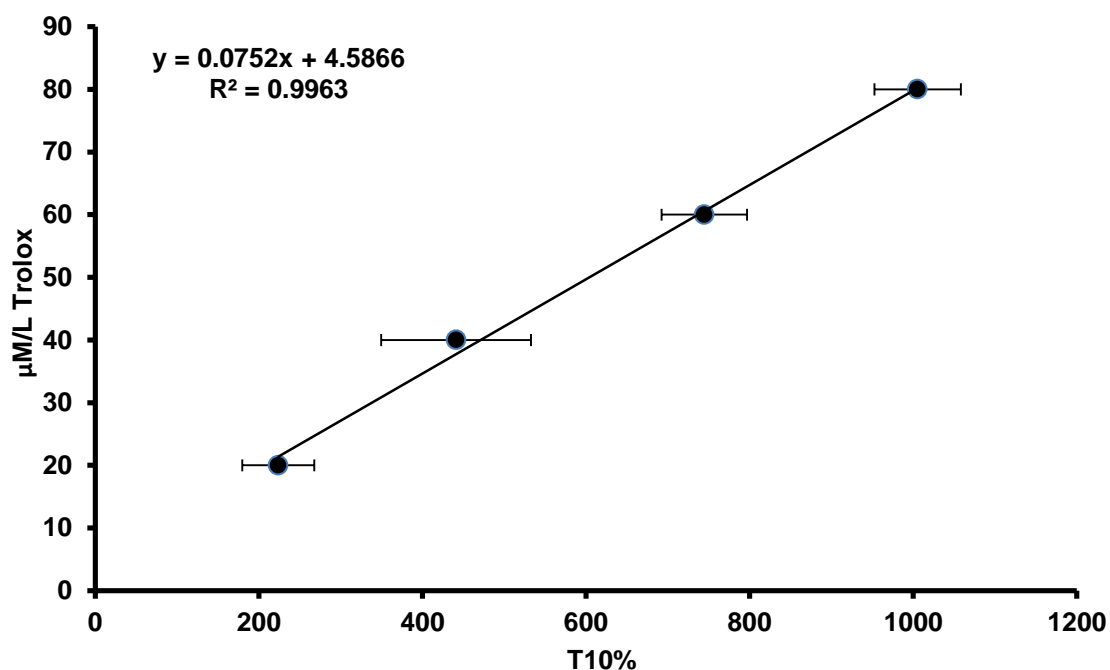
clean glass Rohern tube, along with 1ml running buffer (PBS containing 50mg/L BSA) and the solutions mixed 3 times using manual pipette aspiration. The HRP working solution (1:200 in PBS-BSA; The Binding Site AP311) was subsequently mixed by gently flicking the base of the tube 5 times, manually-aspirated a further 5 times to ensure a good re-mix, and 3-8µl of this working solution added to the signal/buffer mix and further aspirated 5 times, and vortex-mixed briefly (1-2s only). The tube and contents were then loaded immediately into the luminometer and light output measured in real-time until equilibration between 7-9.9V was achieved and began to decrease (typically ~3 minutes). At this stage the Pico software was paused, the tube removed from the luminometer and 20µl of either the Trolox standard, CRP sample (0.2 & 4µg/ml final concentrations) or alternatively azide-free Tris buffer were added. The solutions were mixed thoroughly once again by manual pipette aspiration 5-times and flicking the base of the tube 5-times, and the tube returned immediately to the luminometer and light output recording resumed. A typical recovery curve for the inhibition and restoration of light output by the Trolox standards is shown in Figure 2.9. The recovery time to 10% of the chemiluminescent signal (T10%) was plotted for each Trolox standard and linearity determined as shown in Figure 2.10.

The ability of azide-free CRP (0.2 & 4µg/ml final concentrations) to reduce luminol chemiluminescence generated by horse radish peroxidase/H<sub>2</sub>O<sub>2</sub> was subsequently determined and compared with vehicle control (azide-free Tris buffer). The data was analysed as the percentage reduction in light output as follows.

$$\frac{(\text{Light output after addition CRP} - \text{light output after addition of buffer}) \times 100}{\text{Light output prior to addition}}$$



**Figure 2.9. Typical recovery curve for four Trolox standards (20, 40, 60 and 80 μM/L) in chemiluminescence assay for antioxidant capacity. Recovery time to 10% chemiluminescent signal equates to T10%.**



**Figure 2.10. Typical standard curve for Trolox as the recovery time to 10% chemiluminescent signal (T10% ± standard deviation). Data representative of triplicate readings at 3 time points throughout the day (n=9).**



#### **2.2.11.2 Removal of sodium azide from CRP**

In order to remove the sodium azide preservative from the CRP, 0.5ml CRP was diluted 1:1 with azide-free Tris buffer (total volume = 1ml; final concentration = 1mg/ml) and dialysed against 1L of sodium azide-free buffer (20mM Tris, pH 7.8, containing 280mM sodium chloride, 5mM calcium chloride; TBS), as was a corresponding blank control (Tris buffer containing 1mg/ml sodium azide diluted 1:1 in buffer minus sodium azide). Dialysis was performed using Spectra/Por® Float-A-Lyser® G2 (1ml, MWCO 3.5-5kDa, Sigma Z726060) over 3 days with three 1L buffer changes per day (nine 1L buffer changes in total) whilst stirring at 4°C.

Dialysis was performed aseptically using sterile technique in order to minimize the risk of contamination due to the removal of the sample preservative. In addition, any contaminating bacteria and most enzymes within the dialysis buffer that could affect CRP would be larger than 5kDa and be unable to pass through the dialysis membrane. In addition, having a large buffer dialysis volume also lowered the concentration that any contaminating products could attain between buffer changes. Both dialysed CRP and dialysed buffer blank were filter-sterilised post-dialysis (4mm diam. regenerated cellulose membrane, pore size 0.2µm; Sigma CLS431212) and stored at 2-8°C for a maximum of 2 weeks prior to use.

This dialysis procedure was thought to be rigorous enough to lower the azide concentration within the CRP sample to levels that would be effectively zero. With 9 changes of buffer (1L volumes) over three days, assuming that dialysis was only 30% effective at each buffer change, the calculated residual concentration of sodium azide within the CRP sample would be  $7.621 \times 10^{-20}$  µg/ml.

CRP concentrations were determined using spectrophotometry pre- and post-dialysis. Concentrations were determined from optical density measurements at 280nm (Jenway 6300) using the extinction coefficient for CRP ( $E_{280}^{0.1\%} = 1.75$ ; Nelson 1991, Clapp 2005).

#### **2.2.11.3 Liquid chromatography-tandem mass spectrometry**

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed in order to detect the presence of protein contaminants within the commercial CRP sample. Apart from

initial preparation of azide-free CRP, all procedures were performed by Dr Andy Creese at the School of Biosciences, University of Birmingham. Briefly, after reduction and alkylation of disulphide bonds using dithiothreitol and iodoacetamide, neat CRP samples were digested overnight with trypsin Gold (500ng, Promega, USA) at 37°C and the resultant peptides prepared for analysis as previously described (Grant *et al.* 2010). Peptides were loaded onto a 150mm Acclaim PepMap100 C18 column (LC Packings, Sunnyvale, CA) in mobile phase A (0.1% formic acid. JT Baker, Holland, Sigma Aldrich) and separated over a linear gradient from 3.2% to 44% mobile phase B (acetonitrile + 0.1% formic acid. JT Baker, Sigma) with a flow rate of 350nl/min. The column was then washed with 90% mobile phase B before re-equilibrating at 3.2% mobile phase B. The column oven was heated to 35°C. The LC system was coupled to an Advion Triversa Nanomate (Advion, Ithaca, NY) which infused the peptides with a spray voltage of 1.7kV.

The mass spectrometer performed a full FT-MS scan ( $m/z$  380-1,600) and subsequent collision induced dissociation (CID) MS/MS scans of the five most abundant ions above a threshold of 1,000. Survey scans were acquired in the Orbitrap with a resolution of 60,000 at  $m/z$  400. Precursor ions were subjected to CID in the linear ion trap. The width of the precursor isolation window was 2  $m/z$  and only multiply charged precursor ions were subjected to CID. CID was performed with helium gas at a normalized collision energy of 35% (target  $5 \times 10^4$ , maximum fill time 100ms). CID activation was performed for 10ms. A dynamic exclusion repeat count was set to 1 with a duration of 30s. Data acquisition was controlled by Xcalibur 2.1 (Thermo Fisher Scientific). Purity of CRP was assessed by comparing the intensity of the peptide precursor ions identified from CRP to the peptides which were identified from contaminants.

#### **2.2.11.4 Analytical ultracentrifugation**

Analytical ultracentrifugation was performed in order to assess the molecular structure of commercial CRP both pre- and post-dialysis, along with confirming aggregation of IgG as indicated. Apart from preparation of the protein samples, all procedures were performed by Professor Tim Dafforn at the School of Biosciences, University of Birmingham. A Beckman XL-I analytical ultracentrifuge using an 8-piece An-60 Ti cell rotor in a Beckman-Optima XL-I (Beckman Coulter, ProteomeLab XL-1, Protein Characterisation System) was used for

the analytical ultracentrifugation (AUC) analyses. Protein samples (CRP, IgG and heat aggregated IgG; 450µl) were loaded into 2-sector cells for sedimentation velocity experiments and centrifuged at 32,256rcf for 8hrs at 20°C. The absorbance of the sample was measured at a wavelength of 220nm throughout the cell. A total of 100 measurements were taken throughout each 8hr run. Sedimentation velocity data was analysed using direct boundary Lamm fits from the continuous  $c(s)$  distribution model implemented within SEDFIT (version 11.0) (Schuck 2000). SEDFIT resulted in size- distribution analyses  $c(s)$  that assume that all macromolecular species have the same frictional ratio,  $f/f_0$ .

#### **2.2.11.5 Direct effect of Tris buffer on chemiluminescence**

Assays were performed without neutrophils in pre-blocked (PBS 1% BSA, overnight at 4°C) white microwells (Microlite2, Dynex, East Grinstead, W.Sussex, UK). After washing the plate in PBS, GPBS was added to each well (100µl) followed by luminol (30µl), isoluminol (60µl), lucigenin (30µl) or PBS control (30µl). The plate was then placed into the microplate reader (Bertold microplate luminometer LB96v) at 37°C. Light output was monitored for each well before (2s), during and after (10s; 12s in total) automatic injection of freshly prepared Tris diluted in distilled water (Sigma 93377, pH 7.8-8.2, 0.25-25mM; 40µl). All analyses were performed in quadruplicate and peak light emission in relative light units (RLU) determined.

#### **2.2.11.6 Effect of Tris buffer on H<sub>2</sub>O<sub>2</sub>-induced chemiluminescence**

Assays were performed without neutrophils in pre-blocked (PBS 1% BSA, overnight at 4°C) white microwells (Microlite2, Dynex, East Grinstead, W.Sussex, UK). After washing the plate in PBS, GPBS was added to each well (110µl) followed by luminol (30µl), isoluminol (60µl) with horseradish peroxidase (HRP,  $7.8 \times 10^{-4}$ U; 10µl, Sigma, Gillingham, Kent, UK) or lucigenin (30µl) along with various concentrations of Tris diluted in distilled water Sigma 93377, pH 7.8-8.2, 0.25-25mM; 20µl). The plate was then placed into the microplate reader (Bertold microplate luminometer LB96v) at 37°C. Light output was monitored for each well before (2s), during and after (10s; 12s in total) automatic injection of freshly prepared H<sub>2</sub>O<sub>2</sub> solution (30µl, 3%). All analyses were performed in sextuplicate (6 wells for each substrate) and peak light emission in relative light units (RLU) determined.

#### **2.2.11.7 Effect of CRP and sodium azide on neutrophil viability**

The viability of neutrophils treated with commercial CRP solutions in 20mM Tris, pH 7.8-8.2, containing 280mM sodium chloride, 5mM calcium chloride and 0.1% sodium azide buffer (1-6µg/ml; 37°C) following a 60 minute incubation period was assessed by trypan blue dye exclusion along with cell metabolic activity analysed separately using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Southhampton, UK) with a microplate reader (Bertold microplate luminometer LB96v) (section 2.2.3.6).

#### **2.2.11.8 Neutrophil ROS generation and release in the presence of CRP**

Enhanced chemiluminescence assays were performed as previously described (section 2.2.8). In brief, 100µl of neutrophil suspension ( $1 \times 10^6$  cells/ml in GPBS;  $1 \times 10^5$  cells) were placed in pre-blocked (PBS BSA 1%, overnight at 4°C) white microwells (Microlite 2; Thermo Scientific, Dynex) along with GPBS (20µl), luminol (3mmol/l; pH 7.3; 30µl) or lucigenin (0.33mg/ml; 30µl) and PBS (15µl), or alternatively isoluminol (3mmol/l; 60µl) with 1.5U HRP in PBS (Sigma P8415; 15µl; total volume 75µl). The plate was then carefully and immediately transferred to a microplate reader (37°C, Berthold microplate luminometer; LB96v) and baseline light output was recorded for 1s per well in relative light units (RLU) for 30mins.

Following the initial 30mins baseline measurements, dialysed CRP (10µl) was added (final concentrations: 0, 1, 3 and 10µg/ml in dialysed Tris buffer) and light output recorded in RLU for a further 30 minutes. Following the 30 minute incubation with CRP, neutrophils were subsequently stimulated with either *F. nucleatum* (ATCC 10953; MOI 100:1; 25µl; section 2.2.4.7), opsonized *S. aureus* (NCTC 6571; MOI 300:1; 25µl; section 2.2.4.8), heat-aggregated IgG (25µl; 600µg/ml final well concentration; Sigma I4506; section 2.2.5.1) or PBS (25µl) and light output monitored for an additional 120 minutes. All analyses were performed in triplicate, with peak light emission determined in RLU for the equilibrium phase (“unstimulated”, baseline radical generation), after addition of CRP and after stimulation.

#### **2.2.12 Statistical analyses**

Data for all experiments was manipulated in Microsoft Excel, and evaluated using Minitab (version 15.1.0.0; Minitab Ltd, Coventry, UK) or GraphPad Instat (version 3.10; GraphPad

Software Inc, California, USA). Distributions of all datasets were initially assessed using the Kolmogorov-Smirnov test in order to determine Gaussian distributions, along with effective pairing determined by calculation of the correlation coefficient. The most appropriate statistical test was then applied as outlined (Table 2.5).

	Paired datasets		Unpaired datasets	
	Single	Multiple	Single	Multiple
<b>Parametric</b>	Paired T test	ANOVA with post Tukey test	Unpaired T test	ANOVA with post Tukey test
<b>Non-parametric</b>	Wilcoxon	Friedman with post Dunn's test	Mann-Whitney	Kruskal-Wallis with post Dunn's test

**Table 2.5. Overview of statistical analyses performed depending on the data distribution, pairing and the number of comparisons.**

Unpaired statistical testing comparing patients with chronic periodontitis and controls were deemed the most appropriate analyses given the limitations of patient/control pairing which did not take into consideration certain parameters such as volunteer body mass index (BMI). However paired statistical testing using the Wilcoxon rank sum test was required for the superoxide release data (Chapter 5). Detection of ROS by chemiluminescence methods can show considerable day-to-day variation making the inclusion of a paired, age and gender matched control, whose neutrophils are analysed simultaneously with those from the patient, important if consistent and comparable results are to be obtained (Yaffe *et al.* 1999). Data derived from such experiments are thus normally analysed using the Wilcoxon (one-tailed) test to determine the presence of neutrophil hyper-reactivity (Fredriksson *et al.* 1998, 2003, Matthews *et al.* 2007, Wright *et al.* 2008).

In addition, image capture and analyses for the chemotaxis assay were performed as previously described (section 2.2.10.2) with chemotactic index, cell velocity and speed calculated in Microsoft Excel using pre-determined equations. MATLAB (MathWorks Inc, Massachusetts, USA) was used to produce the various chemotaxis plots (Sapey *et al.* 2011). A level of  $P < 0.05$  was used for assigning statistical significance for all experiments.

# **CHAPTER 3**

## **RESULTS & DISCUSSION**

**Case-control longitudinal intervention study:**  
**Clinical measures & plasma hsCRP levels**

### **3.1 INTRODUCTION**

Patients diagnosed with chronic periodontitis, together with periodontally and systemically healthy control volunteers, were recruited. Baseline clinical measures of periodontal disease were measured for patients and healthy control volunteers to confirm the presence and absence of disease. Additional collection of clinical measures for the patient volunteers was performed post-periodontal treatment to determine treatment effectiveness and to support the observed differences in *ex vivo* neutrophil function.

### **3.2 VOLUNTEER RECRUITMENT TO LONGITUDINAL CLINICAL STUDY**

Patient volunteers (n=20) for the longitudinal case-control interventional study were recruited from those referred to the Periodontal Department of Birmingham Dental Hospital by their General Dental Practitioner (section 2.1.1.3). Once a volunteer diagnosed with chronic periodontitis was recruited, a corresponding age- and gender-matched periodontally and systemically healthy control volunteer was recruited from Staff of Birmingham Dental Hospital and School.

Unfortunately patient volunteer no. 13 decided that she no longer wished to undergo periodontal treatment during the course of root surface debridement and following collection of baseline samples. Therefore, this patient was discontinued from the study and discharged back to her General Dental Practitioner for additional treatment and follow-up as appropriate. All other volunteers (38 in total; 19 pairs) progressed through the clinical study uneventfully.

#### **3.2.1 Age and gender of recruited clinical study volunteers**

Gender distribution comprised of 12 male and 8 female volunteer pairs. Initially patient and control volunteers were matched to within  $\leq 5$  yrs, but because of the limited numbers of periodontally-healthy controls available this was not possible for 6 of the patient and control pairs with the largest difference being 9 yrs for volunteer pair no. 19. Overall mean patient volunteer age to the nearest year at the time of sampling ( $46 \pm 8$  yrs; range 37-61 yrs) was not significantly different to that of the healthy control group ( $46 \pm 8$  yrs; range 32-62 yrs; Mann-Whitney test  $P=0.98$ ; Table 3.1).

Volunteer no.	Gender	Age (yrs)	
		Patient	Control
1	Male	42	43
2	Male	45	41
3	Male	40	44
4	Female	56	62
5	Male	42	41
6	Male	46	47
7	Female	47	50
8	Male	39	32
9	Male	38	38
10	Female	38	42
11	Male	59	55
12	Male	37	45
13	Female	48	42
14	Female	61	59
15	Male	51	58
16	Female	42	39
17	Female	37	35
18	Female	52	52
19	Male	57	48
20	Male	48	48
Mean		46	46
St Dev		8	8
Min		37	32
Max		61	62

**Table 3.1.** Age (to the nearest year at time of sampling) and gender of patient and control volunteers recruited to the longitudinal interventional study.

### 3.3 CLINICAL MEASURES OF PERIODONTAL DISEASE

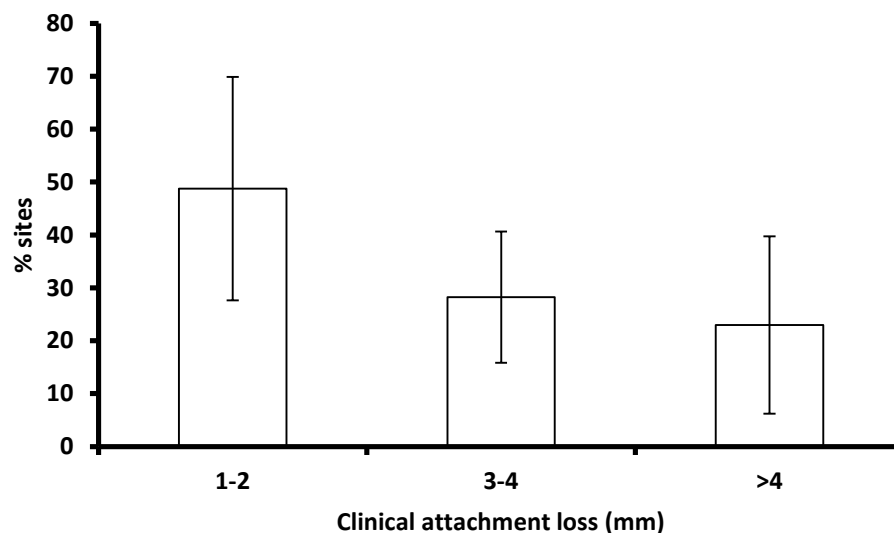
Periodontal probing pocket depths (PPD) and gingival recession were measured and clinical attachment loss calculated (section 2.1.1.4e). Periodontal pocketing >4mm was derived from the raw PPD data, with >2 non-adjacent sites of pocketing >4mm indicative of the presence of periodontal disease in accordance with European Federation of Periodontology guidelines (section 2.1.1.3). As part of the detailed periodontal assessment, the percentage of full-mouth periodontal sulcus/pocket depths demonstrating bleeding on probing (BOP) was calculated (section 2.1.1.4f) and full-mouth gingival (section 2.1.1.4a) and plaque (section 2.1.1.4b)



indices performed. Following the collection of clinical measures, all volunteers were re-appointed for peripheral blood sampling including the preparation of plasma samples for high-sensitivity CRP (hsCRP) analyses (sections 2.1.1.5a and 2.2.2).

### 3.3.1 Clinical attachment loss

Baseline clinical attachment loss (CAL) for individual patient volunteers was measured. Mean CAL and percentage sites with 1-2, 3-4 and >4mm CAL, relating to mild, moderate and severe disease accordingly, along with disease classification are shown in Table 3.2 and Figure 3.1. The majority of patient volunteers presented with generalised moderate and localised severe chronic periodontitis (n=12; 60%), along with generalised mild and localised severe (n=3; 15%) and generalised severe (n=5; 25%) chronic periodontitis.



**Figure 3.1. Severity and extent of baseline clinical attachment loss for patient volunteers (n=20).** Histogram shows mean percentage sites CAL $\pm$ SD.

Patient volunteer no.	Clinical attachment loss				Classification of chronic periodontitis
	Mean $\pm$ SD (mm)	% sites 1-2mm	% sites 3-4mm	% sites >4mm	
1	4.4 $\pm$ 3.2	39.1	21.8	39.1	Generalised severe
2	2.4 $\pm$ 1.9	66.7	26.3	7.0	Generalised moderate, localised severe
3	3.7 $\pm$ 2.1	36.5	35.3	28.2	Generalised moderate, localised severe
4	3.5 $\pm$ 1.8	35.9	41.0	23.1	Generalised moderate, localised severe
5	2.8 $\pm$ 1.6	59.0	24.4	16.6	Generalised moderate, localised severe
6	3.7 $\pm$ 2.1	37.0	31.2	31.8	Generalised severe
7	3.4 $\pm$ 2.4	55.6	21.6	22.8	Generalised moderate, localised severe
8	2.4 $\pm$ 0.9	67.9	28.6	3.5	Generalised moderate, localised severe
9	3.4 $\pm$ 1.6	36.9	45.8	16.3	Generalised moderate, localised severe
10	2.8 $\pm$ 1.3	44.4	48.3	7.2	Generalised moderate, localised severe
11	2.2 $\pm$ 1.8	85.7	5.4	8.9	Generalised mild, localised severe
12	2.9 $\pm$ 2.6	66.1	13.3	20.6	Generalised moderate, localised severe
13	6.0 $\pm$ 2.7	5.8	33.3	60.8	Generalised severe
14	3.3 $\pm$ 2.0	46.4	34.5	19.0	Generalised moderate, localised severe
15	3.9 $\pm$ 1.6	20.1	50.3	29.6	Generalised moderate, localised severe
16	3.2 $\pm$ 2.2	55.7	25.9	18.4	Generalised moderate, localised severe
17	5.9 $\pm$ 3.3	25.6	11.9	62.5	Generalised severe
18	2.2 $\pm$ 1.5	78.0	16.7	5.3	Generalised mild, localised severe
19	4.1 $\pm$ 2.5	33.3	33.3	33.3	Generalised severe
20	1.9 $\pm$ 1.4	79.0	15.6	5.4	Generalised mild, localised severe
Mean	3.4	48.7	28.2	23.0	
SD	1.1	21.1	12.4	16.8	

**Table 3.2.** Clinical attachment loss and classification of chronic periodontitis for patient volunteers at baseline (n=20).

### 3.3.2 Periodontal probing pocket depths

Probing pocket depths for individual patient volunteers pre- and post-treatment and matched healthy controls were measured. Mean PPD and the number of sites >4mm per volunteer is shown (Table 3.3). Whilst the raw PPD data conformed to a normal distribution, this was not confirmed for the number of sites >4mm.

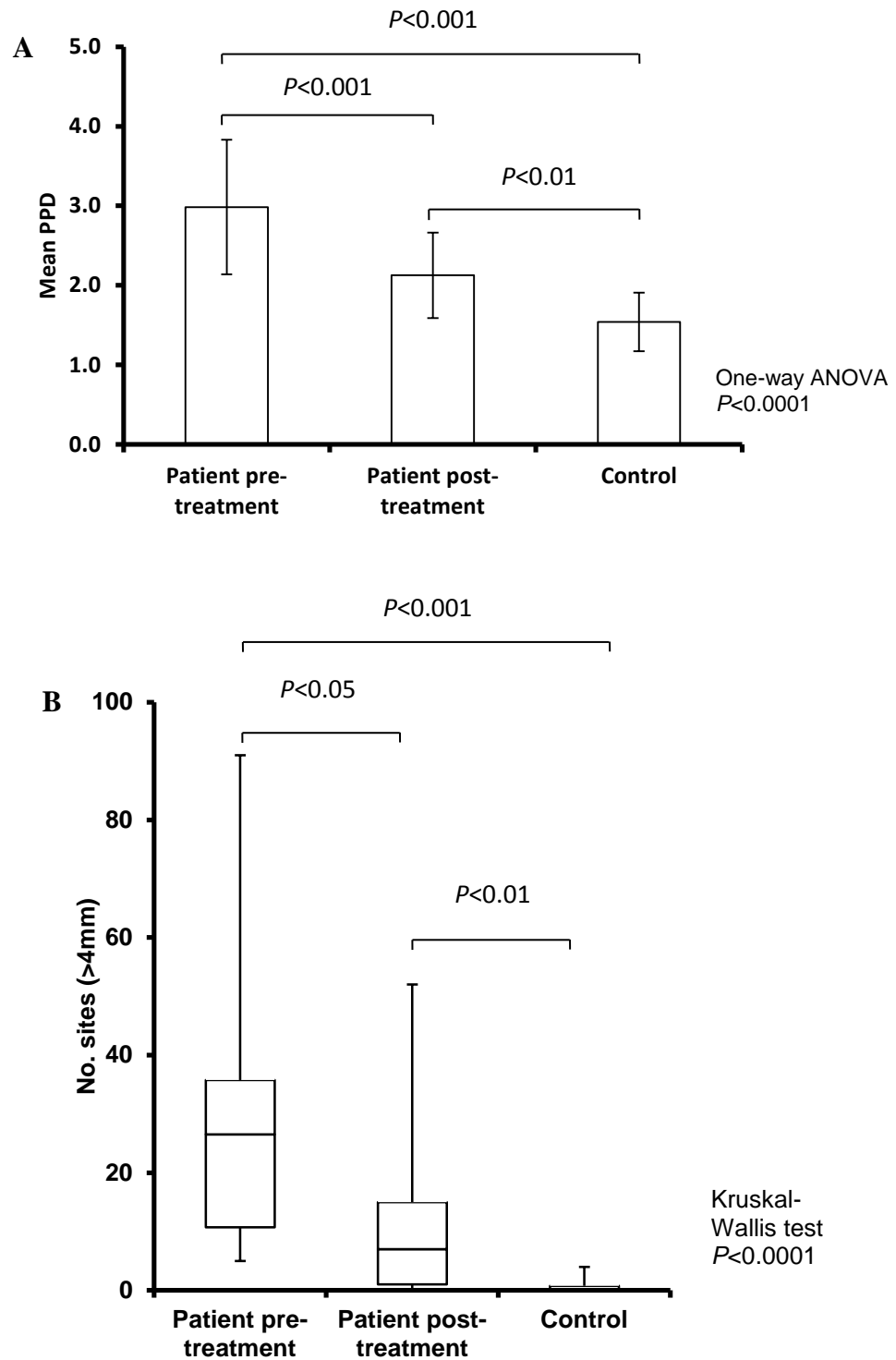
The mean PPD differed between patient volunteers pre- and post-treatment and healthy controls ( $P<0.0001$ ; Figure 3.2A). As expected, baseline PPD in patients ( $3.0\pm0.8\text{mm}$ ) was significantly greater than periodontally healthy controls ( $1.5\pm0.4\text{mm}$ ;  $P<0.001$ ). At the 2-month post-treatment review, the mean PPD in patients ( $2.1\pm0.5\text{mm}$ ) was significantly reduced from their paired baseline, pre-treatment levels ( $3.0\pm0.8\text{mm}$ ;  $P<0.001$ ), although they remained significantly greater than those detected in the healthy control volunteers ( $P<0.01$ ).

Similarly, the number of sites with a PPD >4mm differed between healthy controls and patients pre- and post-treatment ( $P<0.0001$ ; Figure 3.2B). As expected, the baseline median number of sites with a PPD >4mm in patients (27; range 5-91) was significantly greater than periodontally healthy controls (0; range 0-4;  $P<0.001$ ). At the 2-month post-treatment review, the median number of PPD >4mm in patients (7; range 0-52) was significantly reduced from their paired baseline, pre-therapy levels (27; range 5-91;  $P<0.05$ ) although they remained significantly greater than those detected in the healthy control volunteers ( $P<0.01$ ).

One patient displayed an uncharacteristically large number of PPD >4mm of 91 and 52 sites pre- and post-treatment (volunteer no. 17; Table 3.3). This patient subsequently underwent an additional course of periodontal treatment with adjunctive antimicrobials. Omission of the PPD data for volunteer no. 17 did not alter any of the probing pocket depth statistical comparisons.

Volunteer no.	Probing pocket depths								
	Patient pre-treatment			Patient post-treatment			Healthy control		
	Mean $\pm$ SD (mm)	Range (mm)	No. sites >4mm	Mean $\pm$ SD (mm)	Range (mm)	No. sites >4mm	Mean $\pm$ SD (mm)	Range (mm)	No. sites >4mm
1	3.5 $\pm$ 2.8	1-12	45	2.7 $\pm$ 1.4	1-9	24	2.1 $\pm$ 0.9	1-5	1
2	2.2 $\pm$ 1.5	1-12	7	2.2 $\pm$ 0.9	1-7	8	2.1 $\pm$ 0.9	1-5	1
3	3.4 $\pm$ 2.0	1-9	36	2.7 $\pm$ 1.3	1-7	15	1.8 $\pm$ 0.7	1-4	0
4	3.4 $\pm$ 1.7	1-9	33	1.6 $\pm$ 0.7	0-4	0	2.0 $\pm$ 0.8	1-5	3
5	2.6 $\pm$ 1.5	1-7	22	2.0 $\pm$ 0.8	0-5	2	1.2 $\pm$ 0.4	1-2	0
6	3.4 $\pm$ 2.1	1-8	45	2.5 $\pm$ 1.0	1-7	7	1.5 $\pm$ 0.6	1-3	0
7	2.9 $\pm$ 1.9	1-12	28	2.2 $\pm$ 1.4	1-10	9	1.7 $\pm$ 0.6	1-4	0
8	2.3 $\pm$ 0.9	1-7	5	1.4 $\pm$ 0.8	0-5	1	2.2 $\pm$ 0.9	1-5	4
9	3.4 $\pm$ 1.6	1-8	29	2.4 $\pm$ 1.1	1-6	7	1.3 $\pm$ 0.5	1-3	0
10	2.6 $\pm$ 1.2	1-7	10	1.8 $\pm$ 0.9	1-4	0	1.4 $\pm$ 0.6	1-3	0
11	2.1 $\pm$ 1.8	1-9	14	1.5 $\pm$ 1.1	0-9	2	1.1 $\pm$ 0.4	1-3	0
12	2.8 $\pm$ 2.4	0-12	35	2.5 $\pm$ 2.1	1-10	23	1.1 $\pm$ 0.4	1-3	0
13	5.0 $\pm$ 2.6	1-13	54	Treatment discontinued			1.1 $\pm$ 0.3	1-2	0
14	2.9 $\pm$ 1.7	0-8	25	1.8 $\pm$ 1.2	0-6	5	1.8 $\pm$ 0.9	1-5	1
15	2.8 $\pm$ 1.4	1-7	23	2.4 $\pm$ 1.5	1-8	16	1.3 $\pm$ 0.5	1-3	0
16	3.2 $\pm$ 2.2	1-9	32	2.0 $\pm$ 1.3	0-9	9	1.1 $\pm$ 0.3	1-3	0
17	4.8 $\pm$ 2.7	1-12	91	3.5 $\pm$ 2.2	1-10	52	1.4 $\pm$ 0.5	1-3	0
18	2.1 $\pm$ 1.5	0-11	8	1.5 $\pm$ 0.9	0-7	1	1.7 $\pm$ 0.7	1-4	0
19	2.8 $\pm$ 1.6	1-9	13	2.2 $\pm$ 1.3	1-9	8	1.6 $\pm$ 0.6	1-3	0
20	1.5 $\pm$ 1.0	1-7	5	1.5 $\pm$ 0.8	1-7	1	1.3 $\pm$ 0.6	1-4	0
Mean	3.0			2.1			1.5		
SD	0.8			0.5			0.4		
Max			91			52			4
Q3			36			15			1
Median			27			7			0
Q1			11			1			0
Min			5			0			0

**Table 3.3. Probing pocket depths for patients (n=20 pre-treatment; n=19 post-treatment) and periodontally and systemically healthy control volunteers (n=20).** Data presented as probing pocket depths (mean  $\pm$  SD & range) and the number of sites >4mm.



**Figure 3.2. Probing pocket depths (A) and number of sites >4mm (B) in patients and matched periodontally and systemically healthy control volunteers (n=19).** Histogram (A) shows mean PPD $\pm$ SD, &  $P$ -values (Tukey-Kramer multiple comparisons test). Box and whisker plot (B) shows median, interquartile range, maximum and minimum values &  $P$ -values (Dunn's multiple comparisons test).

### **3.3.3 Bleeding on periodontal probing**

Bleeding on probing was recorded and the percentage of sites displaying BOP calculated for each individual volunteer during the sampling visits (Table 3.4; Figure 3.3). As expected, the baseline median percentage of sites displaying BOP in patients (43; range 16-87) was significantly greater than periodontally healthy controls (2; range 0-39;  $P<0.001$ ) consistent with the presence of periodontal inflammation. At the 2-month post-treatment review, the median percentage of sites with BOP in patients (14; range 3-35) was significantly reduced from their paired baseline pre-therapy levels (43; range 16-87;  $P<0.001$ ; Figure 3.3). Post-therapy BOP levels were not significantly different from healthy controls, despite patients continuing to have a greater median percentage of sites that displayed BOP ( $P>0.05$ ).

### **3.3.4 Gingival indices**

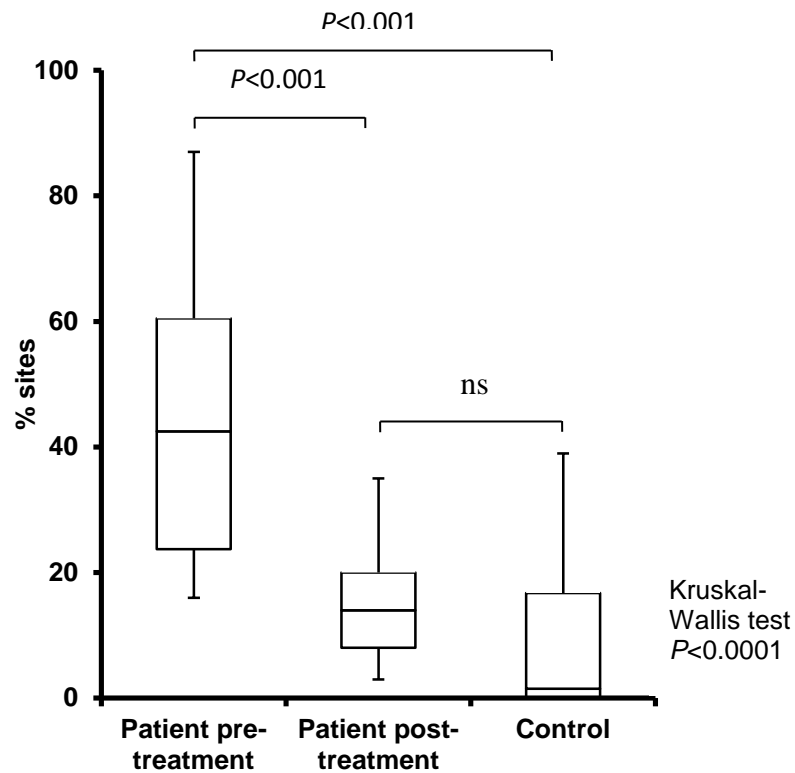
Individual and full-mouth cumulative gingival indices (GI) are shown in Table 3.5. Median and cumulative GI differed between pre- and post-treatment patient volunteers and healthy controls ( $P<0.0001$ ). Baseline median and cumulative GI values in patients were significantly greater than periodontally healthy controls ( $P<0.001$ ) as expected and consistent with the presence of periodontal inflammation. At the 2-month post-treatment review, the GI values in patients were significantly reduced from their paired baseline pre-therapy levels ( $P<0.01$ ) to levels comparable to healthy controls ( $P>0.05$ ; Figure 3.4).

### **3.3.5 Plaque indices**

Individual and full-mouth cumulative plaque indices (PI) are shown in Table 3.6. Median and cumulative PI differed between patient volunteers pre- and post-treatment and healthy controls ( $P<0.0001$ ). Baseline median and cumulative PI values were significantly greater than periodontally healthy controls ( $P<0.001$ ). At the 2-month post-treatment review, the PI values in patients were significantly reduced from the paired baseline pre-therapy levels ( $P<0.001$ ) to levels comparable to healthy controls ( $P>0.05$ ; Figure 3.5).

Volunteer no.	Bleeding on probing (% sites)		
	Patient		Healthy control
	Pre-treatment	Post-treatment	
1	37	23	39
2	16	14	17
3	87	33	0
4	51	8	0
5	26	5	0
6	47	12	0
7	44	11	16
8	42	18	20
9	72	15	0
10	63	13	1
11	21	10	0
12	53	22	0
13	41	Discontinued	0
14	70	14	20
15	23	20	2
16	21	5	0
17	63	35	9
18	33	5	16
19	43	14	17
20	17	3	8
Max	87	35	39
Q3	61	20	17
Median	43	14	2
Q1	24	8	0
Min	16	3	0

**Table 3.4. Percentage sites displaying bleeding upon periodontal probing for patients (n=20 pre-treatment; n=19 post-treatment) and periodontally and systemically healthy control volunteers (n=20).** Data presented as percentage sites with bleeding on periodontal probing.

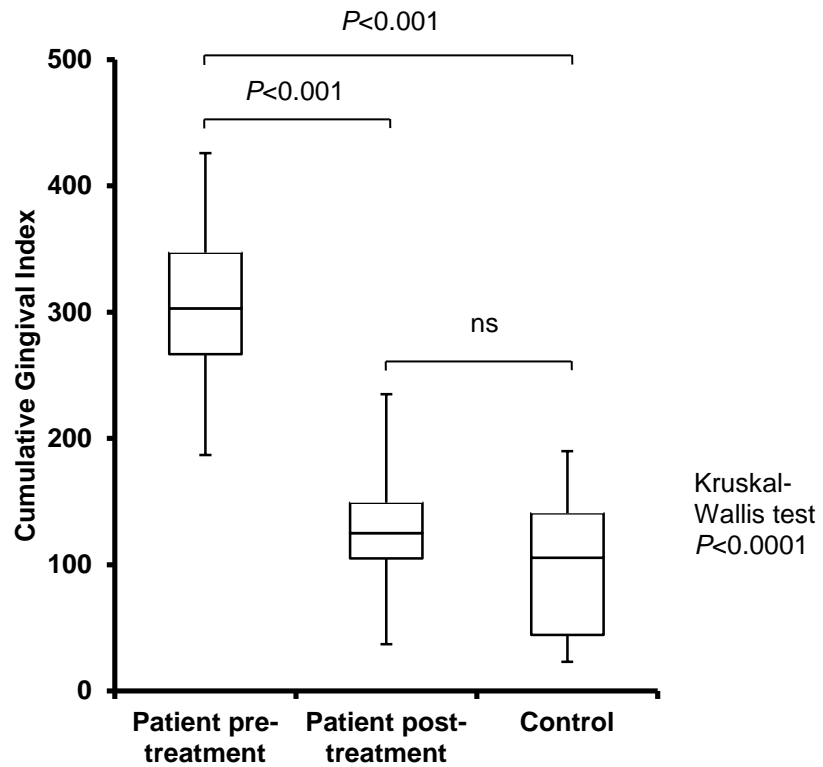


**Figure 3.3. Percentage of sites demonstrating bleeding on periodontal probing in patients and matched periodontally and systemically healthy control volunteers (n=19).** Box and whisker plots showing median, interquartile range, maximum and minimum values &  $P$ -values (Dunn's multiple comparisons test).




Volunteer no.	Gingival index					
	Patient				Healthy control	
	Pre-treatment		Post-treatment			
	Individual	Cumulative	Individual	Cumulative	Individual	Cumulative
1	1 (1-2)	195	1 (0-1)	111	1 (0-1)	136
2	1 (1-2)	187	1 (0-1)	137	1 (0-1)	141
3	3 (2-3)	426	1 (1-2)	216	1 (0-1)	103
4	3 (2-3)	405	1 (1-1)	169	1 (1-2)	190
5	2 (2-2)	322	1 (0-1)	94	0 (0-1)	77
6	2 (2-2)	350	1 (0-1)	149	1 (0-1)	136
7	2 (1-2)	310	1 (1-2)	216	1 (1-1)	185
8	2 (1-2)	316	1 (1-2)	235	0 (0-1)	87
9	2 (1-2)	296	1 (1-1)	126	0 (0-1)	43
10	1 (1-2)	292	1 (0-1)	112	0 (0-0)	26
11	1 (1-1)	203	1 (0-1)	122	0 (0-1)	36
12	2 (1-3)	338	1 (0-1)	125	0 (0-0)	23
13	2 (2-3)	274	Discontinued		0 (0-1)	43
14	2 (1-4)	338	1 (0-1)	106	1 (0-1)	140
15	2 (2-2)	358	0 (0-1)	105	1 (0-1)	108
16	1 (1-2)	274	0 (0-1)	83	0 (0-1)	49
17	2 (2-2)	390	1 (0-1)	147	0 (0-1)	81
18	2 (1-2)	266	0 (0-1)	37	1 (0-1)	108
19	2 (2-2)	269	1 (1-1)	145	1 (1-1)	178
20	1 (1-2)	250	0 (0-1)	72	1 (1-1)	177
Max	3	426	1	235	1	190
Q3	2	347	1	149	1	141
Median	2	303	1	125	1	106
Q1	1	267	1	105	0	45
Min	1	187	0	37	0	23
Friedman test						
P<0.0001						
P<0.0001						
Dunn's test						
P<0.01						
P<0.001						
NS						
P<0.001						
P<0.001						
NS						

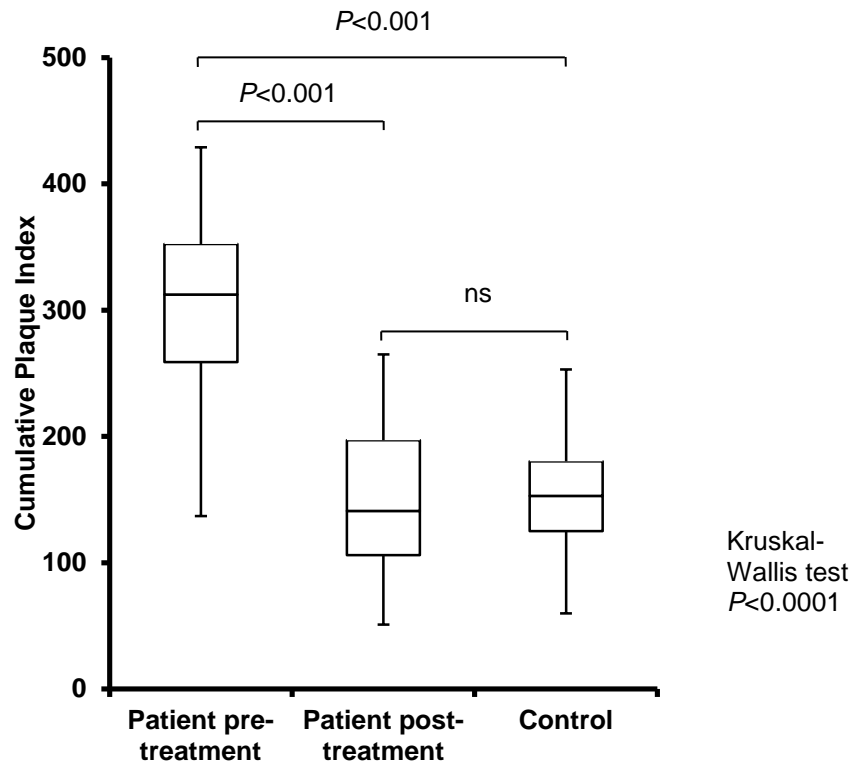
Table 3.5. Gingival indices for patient (n=20 pre-treatment; n=19 post-treatment) and periodontally and systemically healthy control volunteers (n=20). Data presented as individual median gingival index and interquartile range along with full-mouth cumulative values. *P*-values calculated (n=19) using Friedman test followed by Dunn's multiple comparisons test (Groups compared in table = ).



**Figure 3.4. Cumulative gingival index in patients and matched periodontally and systemically healthy control volunteers (n=19).** Box and whisker plots showing median, interquartile range, maximum and minimum values & *P*-values (Dunn's multiple comparisons test).

Volunteer no.	Plaque index					
	Patient				Healthy control	
	Pre-treatment		Post-treatment			
	Individual	Cumulative	Individual	Cumulative	Individual	Cumulative
1	1 (1-2)	210	1 (1-2)	185	1 (1-1)	168
2	1 (1-1)	137	1 (1-2)	197	1 (0-1)	125
3	3 (2-3)	429	2 (1-2)	244	1 (1-1)	182
4	2 (2-3)	382	1 (1-1)	150	2 (1-2)	249
5	2 (2-3)	367	1 (0-1)	141	1 (1-2)	186
6	2 (2-2)	311	1 (1-2)	212	1 (1-2)	253
7	2 (1-3)	351	1 (1-2)	218	1 (1-2)	212
8	2 (1-2)	314	2 (1-2)	265	1 (0-1)	145
9	2 (1-2)	256	1 (1-1)	150	0 (0-1)	60
10	1 (1-2)	228	1 (0-1)	100	1 (1-1)	157
11	1 (1-2)	250	0 (0-1)	73	1 (1-1)	119
12	2 (1-2)	267	1 (0-1)	117	0 (0-1)	61
13	3 (3-4)	345	Discontinued		1 (0-1)	124
14	2 (2-3)	339	1 (1-2)	135	1 (1-2)	156
15	2 (2-3)	353	1 (1-1)	116	1 (0-1)	128
16	2 (1-2)	272	0 (0-1)	51	1 (0-1)	125
17	2 (1-3)	324	1 (0-1)	106	1 (0-1)	143
18	2 (1-3)	279	0 (0-1)	57	1 (1-1)	150
19	2 (2-3)	271	1 (1-2)	147	1 (1-1)	175
20	2 (2-3)	366	1 (0-1)	122	1 (1-2)	162
Max	3	429	2	265	2	253
Q3	2	353	1	197	1	180
Median	2	313	1	141	1	153
Q1	2	259	1	106	1	125
Min	1	137	0	51	0	60
Friedman test						
P<0.0001						
P<0.0001						
Dunn's test						
P<0.01						
P<0.001						
NS						
P<0.001						
P<0.001						
NS						

Table 3.6. Plaque indices for patients (n=20 pre-treatment; n=19 post-treatment) and periodontally and systemically healthy control volunteers (n=20). Data presented as median gingival index and interquartile range along with full-mouth cumulative values. \* *P*-values calculated (n=19) using Friedman test (*P*<0.0001) followed by Dunn's multiple comparisons test (Groups compared in table = ).



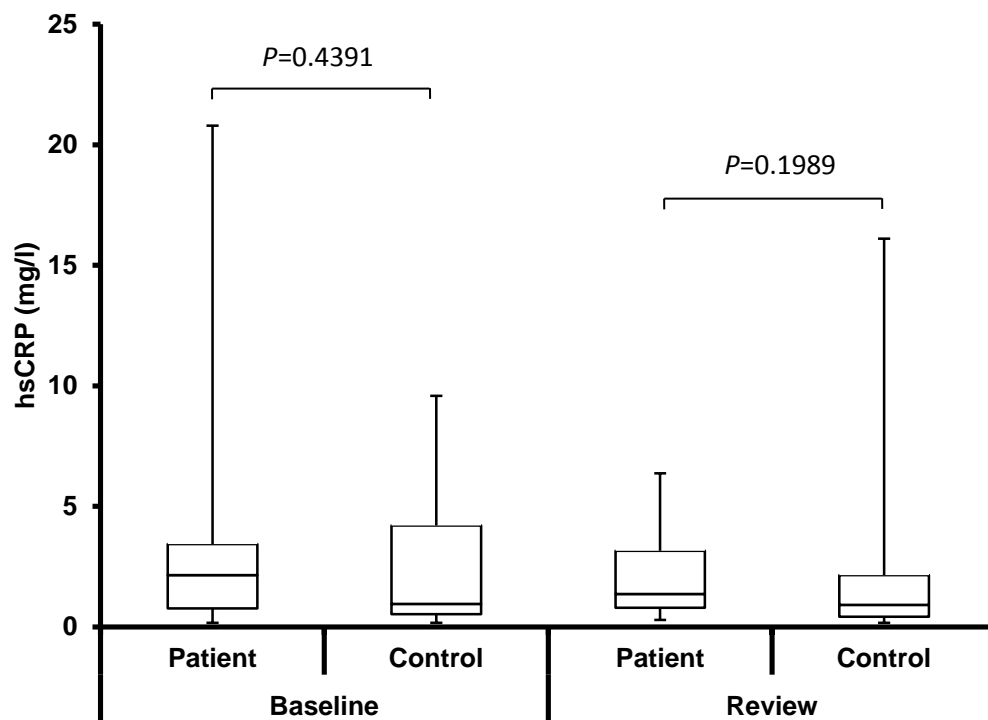
**Figure 3.5. Cumulative plaque index in patients and matched periodontally and systemically healthy control volunteers (n=19).** Box and whisker plots showing median, interquartile range, maximum and minimum values & *P*-values (Dunn's multiple comparisons test).

### 3.4 Plasma high-sensitivity C-reactive protein concentrations pre- and post-periodontal treatment

Plasma hsCRP concentrations are shown in Table 3.7. Median hsCRP concentrations in patients did not significantly differ from those detected in periodontally healthy controls either at baseline or at the 2 month review (Figure 3.6). However, individual variations in plasma hsCRP concentrations were observed between the baseline and review sampling visits (Table 3.7). Notably, patient volunteer no. 4 presented with a high baseline hsCRP concentration (20.80mg/ml) that was reduced markedly at the post-periodontal treatment review (2.69mg/ml). Similarly control volunteer no. 17 had a high review hsCRP concentration (16.10mg/ml) despite a much lower concentration (0.79mg/ml) at the baseline sampling visit.

Volunteer no.	hsCRP (mg/l)			
	Baseline		Review	
	Patient	Control	Patient	Control
1	6.82	0.79	3.16	0.91
2	2.69	0.73	0.74	0.65
3	2.95	2.72	3.56	1.39
4	20.80	0.41	2.69	2.02
5	0.56	9.58	0.28	0.32
6	0.60	1.89	0.99	0.98
7	Insufficient sample*		5.21	0.42
8	5.13	0.53	5.10	0.79
9	3.18	4.42	2.01	3.18
10	1.27	0.95	1.02	0.57
11	0.16	0.18	0.81	0.35
12	0.77	9.12	0.65	0.23
13	5.87	5.41	Discontinued	
14	0.45	4.20	0.79	4.62
15	0.92	1.18	1.36	1.09
16	1.44	0.16	0.89	0.16
17	2.15	0.79	1.80	16.10
18	0.98	3.05	0.74	4.30
19	3.43	0.76	1.97	2.15
20	2.23	0.48	6.37	0.49
Max	20.80	9.58	6.37	16.10
Q3	3.43	4.20	3.16	2.15
Median	2.15	0.95	1.36	0.91
Q1	0.77	0.53	0.79	0.42
Min	0.16	0.16	0.28	0.26

**Table 3.7. Plasma hsCRP concentrations (mg/l) for patients and periodontally and systemically healthy control volunteers at baseline and review (n=19).** \* Due to difficult venous access, limited blood was collected from patient and control volunteer no. 7 and therefore pre-treatment hsCRP levels could not be determined.



**Figure 3.6. Baseline and review plasma hsCRP concentrations for patients and matched periodontally and systemically healthy control volunteers (n=19).** Box and whisker plots showing median, interquartile range and maximum and minimum values. *P*-values calculated using Mann-Whitney test.

### 3.5 DISCUSSION

The majority of patient volunteers recruited to the clinical study were categorised with generalised moderate, localised severe chronic periodontitis (Armitage 1999, Milward & Chapple 2003). All patients had clinical measures of chronic periodontitis consistent with the national Adult Dental Health Survey and previously published literature (Goodson *et al.* 1982, Fuller *et al.* 2011, Chapple *et al.* 2012, Steele *et al.* 2012, White *et al.* 2012). All healthy control volunteers had clinical measures consistent with periodontal health.

Patient volunteers progressed through a course of non-surgical periodontal therapy including root surface debridement and intensive oral hygiene instruction since the plaque biofilm is the initiating (and a modifiable) factor in periodontal disease pathogenesis (L  e *et al.* 1965, Theilade *et al.* 1966). All patients benefited from periodontal treatment as indicated by

individual reductions in all clinical measures associated with clinical periodontitis. However, overall treatment responses varied between patient volunteers. A variety of factors might explain this variation in treatment response, including the individual host response and susceptibility and compliance with prescribed oral hygiene advice (Axelsson & Lindhe 1981, Lang & Tonneti 1996). Several patients achieved stable periodontal treatment outcomes (Sanz *et al.* 2012, Dentino *et al.* 2013) and therefore required no additional corrective periodontal therapy other than periodontal maintenance to reduce the risk of disease recurrence (Chambrone *et al.* 2010). However a number of patient volunteers continued to display clinical measures consistent with continuing chronic periodontitis at review, all of which were reduced in comparison to individual baseline values. For example, patient volunteer no. 17 required additional periodontal treatment because of the persistence of periodontal pocketing. This was despite a marked reduction in periodontal inflammation and plaque deposits confirming patient compliance with prescribed oral hygiene advice. Along with individual host susceptibility and compliance with plaque control, variations in treatment response may also be a reflection of the relative disease severity and extent at initial presentation. If chronic periodontitis is severe with abundant supra- and subgingival plaque and calculus deposits, as was the case with patient volunteer no. 17, additional courses of root surface debridement (Badersten *et al.* 1984), use of local (Matesanz-Perez *et al.* 2013) or systemic antimicrobials (Zandbergen *et al.* 2013) or alternatively, periodontal surgery (Graziani *et al.* 2012) may be required to achieve periodontal stability. Histological studies have confirmed that periodontal disease stabilisation occurs through a combination of attachment gain with the formation of a long junctional epithelium, along with post-treatment gingival recession due to the resolution of gingival inflammation and subsequent collagen cross-linking with tightening of the gingival cuff (Lindhe *et al.* 1982, Nyman *et al.* 1982). Reductions in BOP and GI result from the resolution of inflammation and the repair of sulcular/pocket epithelium ulceration, whereas a reduction in PI results from improved mechanical plaque removal by the patient following compliance with individually tailored oral hygiene instruction.

Median plasma hsCRP concentration for the patient volunteers (2.15mg/l) was within the reported range of 1-4mg/l for patients with periodontitis (Fredriksson *et al.* 1999, Loos *et al.* 2000, Offenbacher *et al.* 2009, D'Aiuto *et al.* 2010, Allen *et al.* 2011). The median plasma hsCRP concentration of periodontally-healthy control volunteers was lower than that detected

for patients pre-treatment, however there was no significant difference when the groups were compared. Furthermore, median plasma hsCRP concentrations in patients were non-significantly reduced by periodontal treatment to levels similar to those of control volunteers. This study may be relatively underpowered with regard to detecting changes in systemic CRP concentration with previously large meta-analyses (Paraskevas *et al.* 2008) required to identify relatively subtle, but statistically significant, differences in systemic CRP observed as a result of chronic periodontitis. Therefore, although the differences were not statistically significant, the trend in plasma hsCRP concentration between patients and controls presented in this thesis is consistent with current literature.

Patient and control volunteers had median plasma hsCRP concentrations of 2.15 and 0.95mg/l respectively, thereby falling within the average and low risk categories respectively for the development of cardiovascular disease according to the American Heart Association guidelines (Ridker *et al.* 2002, 2005; section 1.7.1). With regards to periodontitis and co-morbidity, systemic CRP concentrations have been measured in patients with chronic periodontitis and co-diagnosed with cardiovascular disease (CVD) with CRP concentrations decreasing following stabilisation of periodontal disease (Hussain Bokhari *et al.* 2009, El Fadl *et al.* 2011, Bokhari *et al.* 2012, Koppolu *et al.* 2013, Zhou *et al.* 2013), suggesting that periodontitis is a potential modifiable risk factor for CVD. In addition, systemic CRP concentrations have also been measured in patients co-diagnosed with type-2 diabetes, with serum and GCF CRP concentrations similarly decreasing following periodontal treatment (Sun *et al.* 2010, 2011, Pradeep *et al.* 2013). Reductions in systemic CRP have also been observed in patients diagnosed with type-1 diabetes following periodontal treatment, albeit non-significantly, in agreement with the data presented in this thesis (Correa *et al.* 2010, Kardesler *et al.* 2010, Llambes *et al.* 2012). Systemic CRP concentrations have also been measured in patients with chronic periodontitis co-diagnosed with chronic renal disease and receiving haemodialysis treatment, with CRP levels significantly reducing following periodontal treatment (Kadiroglu *et al.* 2006, Siribamrungwong & Puangpanngam 2012, Yazdi *et al.* 2013). However CRP concentrations did not relate to the severity of periodontitis, possibly due to the large superimposed inflammatory burden as a result of the renal disease, thereby masking the effect of the periodontal disease (Yazdi *et al.* 2013). In addition, systemic CRP concentrations have been measured in patients with chronic periodontitis co-diagnosed



with rheumatoid arthritis (Pinho Mde *et al.* 2009), obesity (Al-Zahrani & Alghamdi 2012) and metabolic syndrome (Acharya *et al.* 2010, Lopez *et al.* 2012) with CRP concentrations significantly decreasing following periodontal treatment for patients with metabolic syndrome but not for rheumatoid arthritis.

Whilst no significant differences in patient and control plasma CRP concentrations are reported in this thesis, systemic CRP concentrations are considered important in investigating co-morbidity links between periodontal and systemic health. The effect of CRP on the host inflammatory-immune response remains to be elucidated, including neutrophil responses such as neutrophil respiratory burst activity. Systemic reactive oxygen metabolites positively associate with plasma CRP concentrations in chronic periodontitis (D'Aiuto *et al.* 2010) suggesting a link between CRP and systemic oxidative stress in the pathogenesis of chronic periodontitis. Therefore, part of this thesis (Chapter 8) has specifically examined the effects of CRP on neutrophil respiratory burst activity along with its antioxidant potential in relation to the host response and the pathogenesis of both chronic periodontitis and the systemic inflammatory burden.

# **CHAPTER 4**

## **RESULTS & DISCUSSION**

**Case-control longitudinal intervention study:  
Neutrophil cytokine release in chronic periodontitis**

## 4.1 INTRODUCTION

Cytokine release from peripheral blood neutrophils isolated from patients with chronic periodontitis pre- and post-treatment was determined and compared to matched healthy controls in order to investigate whether aberrant neutrophil cytokine release is a plausible mechanism in the pathogenesis of chronic periodontitis. Peripheral blood samples were collected from the study volunteers at baseline and review (following non-surgical periodontal treatment of patients) and, after isolation, neutrophils were cultured for 18 hours as previously described (sections 2.2.3 and 2.2.6). Culture supernatants were harvested and the concentrations of IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  were determined by ELISA.

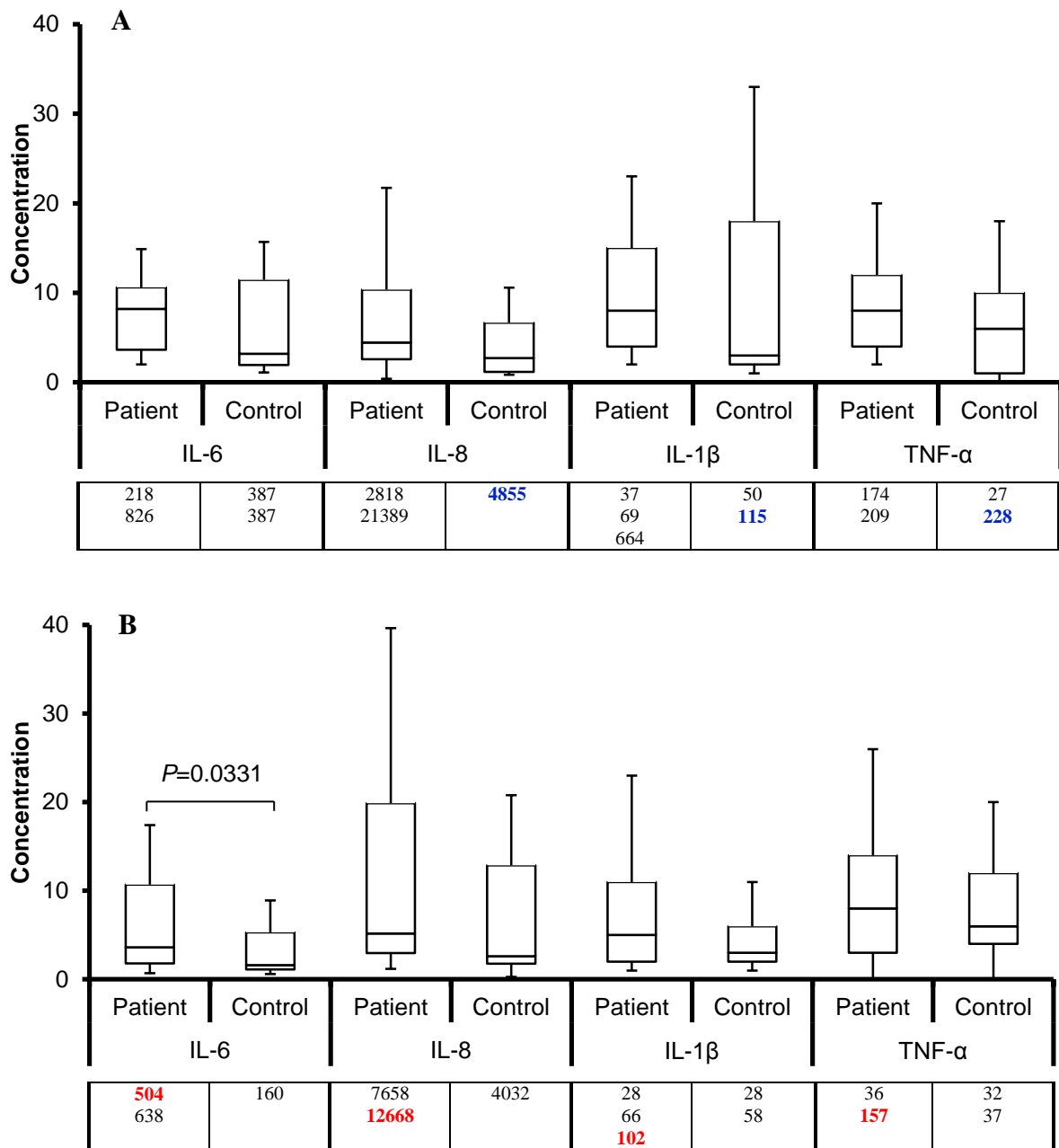
## 4.2 NEUTROPHIL VIABILITY FOLLOWING CULTURE

There were no significant differences in neutrophil viability following 18 hours of culture between patient (mean 97.7%; range 94.1-100%) and control volunteers (mean 97.5%; range 93.5-100%;  $P=0.7983$ ). However, comparing all treatments, there was a significant difference in mean neutrophil viability following 18 hours of culture in the presence of additional RPMI (98.6%), *E. coli* LPS (98.4%), *F. nucleatum* (99.2%), *P. gingivalis* (99.2%) and opsonised *S. aureus* (95.4%) ( $P<0.0001$ ). This difference was due to neutrophil viability in the presence of opsonised *S. aureus* being significantly lower than that in cultures containing additional RPMI or other stimuli ( $P<0.05$ ).

## 4.3 UNSTIMULATED NEUTROPHIL CYTOKINE RELEASE

Neutrophils were cultured in the presence of additional RPMI (vehicle control) to determine cytokine release from essentially unstimulated cells (section 2.2.6; Figure 4.1). At baseline, there were significant differences in the amounts of each cytokine released by neutrophils ( $P<0.0001$ ) after 18 hours culture, with a clear pattern of release (IL-8>IL-6>IL-1 $\beta$ =TNF $\alpha$ ). There was considerable individual variation in cytokine release by both patient and control cells. This was particularly evident for IL-8 release by patients' cells and IL-1 $\beta$  release by both patient and control cells (Figure 4.1A). Overall, median levels of baseline IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  release from patient neutrophils were higher than those from controls, although this was not statistically significant when comparing data for individual cytokines ( $P\leq 0.3335$ ) or data for all cytokines combined ( $P=0.1658$ ).

At review, and as seen at baseline, patient and control neutrophils consistently released amounts of cytokines in the order IL-8>IL-6>IL-1 $\beta$ =TNF $\alpha$  (Table 4.1). Overall, there was greater variation in cytokine release from patient neutrophils at review compared to control cells. This was particularly noticeable for IL-8 due to very high amounts being released from cells derived from 2 donors (Figure 4.1B). As seen at baseline, median levels of cytokine release at review were greater from patient compared to control neutrophils, however this difference was only statistically significant for IL-6 (median 36 & 16pg/ml;  $P=0.0331$ ), with no significant difference for all cytokines combined ( $P=0.1586$ ).



**Figure 4.1. Unstimulated cytokine release (pg/ml, IL-1 $\beta$  & TNF- $\alpha$ ; pg/mlx10<sup>-1</sup>, IL-6; pg/mlx10<sup>-2</sup>, IL-8) by neutrophils (A) baseline, pre-treatment (n=17) and (B) review, post-treatment (n=17) after 18 hours culture in the absence of stimulation (RPMI control). Box and whisker plots showing median, interquartile range, maximum and minimum. Values for outliers (calculated as >1.5xIQR) are tabulated. (A) control volunteer no. 5 gave consistently high values in the absence of stimulation and are highlighted in blue; (B) patient volunteer no. 6 gave consistently high values in the absence of stimulation and are highlighted in red. *P*-values are calculated using two-tailed Mann-Whitney tests.**

#### 4.4 STIMULATED NEUTROPHIL CYTOKINE RELEASE

Neutrophils generally released significantly greater amounts of IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  in the presence of *E. coli* LPS, *F. nucleatum*, opsonised *S. aureus* and *P. gingivalis* compared with RPMI control, demonstrating a positive cytokine response to the stimuli employed ( $P \leq 0.05$ ; Table 4.2; Figures 4.2-4.4). However, *F. nucleatum* did not stimulate significant IL-6 release from patient or control neutrophils, despite median levels being higher than the RPMI control (Figure 4.3). When considering the combined cytokine levels for all stimuli tested, stimulated patient and control neutrophils showed a similar cytokine release profile to that found for unstimulated cells (i.e. IL-8 > IL-6 > TNF- $\alpha$  = IL-1 $\beta$ ), with amounts released differing significantly between cytokines ( $P < 0.0001$ ; Table 4.3). As seen in unstimulated cultures, cytokine levels varied greatly between cells from different individuals. Overall, there was greater variation in cytokine release from patient compared with control neutrophils for all stimulated conditions.

Cytokine	Stimulus	Change in cytokine concentration compared with RPMI control			
		Baseline		Review	
		Patient	Control	Patient	Control
IL-6 (n=17)	<i>E. coli</i> LPS	↑ ***	↑ ***	↑ ***	↑ ***
	<i>F. nucleatum</i>	↔	↔	↔	↔
	ops <i>S. aureus</i>	↑ **	↑ ***	↑ ***	↑ ***
	<i>P. gingivalis</i>	↑ ***	↑ ***	↑ ***	↑ **
IL-8 (n=18)	<i>E. coli</i> LPS	↑ **	↑ ***	↑ ***	↑ ***
	<i>F. nucleatum</i>	↑ ***	↑ ***	↑ ***	↑ ***
	ops <i>S. aureus</i>	↑ ***	↑ ***	↑ ***	↑ ***
	<i>P. gingivalis</i>	↑ ***	↑ ***	↑ ***	↑ ***
IL-1β (n=19)	<i>E. coli</i> LPS	↑ ***	↑ ***	↑ ***	↑ ***
	<i>F. nucleatum</i>	↑ *	↑ ***	↑ *	↑ **
	ops <i>S. aureus</i>	↑ ***	↑ ***	↑ ***	↑ ***
	<i>P. gingivalis</i>	↑ **	↑ *	↑ **	↑ *
TNF-α (n=19)	<i>E. coli</i> LPS	↑ ***	↑ ***	↑ ***	↑ ***
	<i>F. nucleatum</i>	↑ ***	↑ ***	↑ ***	↑ ***
	ops <i>S. aureus</i>	↑ ***	↑ ***	↑ ***	↑ ***
	<i>P. gingivalis</i>	↑ *	↑ *	↑ **	↑ *

**Table 4.1. Summary of the difference in cytokine release by neutrophils after 18 hours culture in the presence of *E. coli* LPS (5µg/ml), *F. nucleatum* (MOI 100:1), IgG-opsonised *S. aureus* (MOI 300:1) and *P. gingivalis* (MOI 100:1) compared to RPMI control. ↑ signifies increase and ↔ signifies no change. *P*-values calculated using Dunn's multiple comparisons test; \* *P*<0.05, \*\* *P*<0.01, \*\*\* *P*<0.001.**

Cytokine	Patient		Control		Comparison between cytokines		
	Baseline	Review	Baseline	Review	Kruskal-Wallis	Dunn's test	
IL-8	20,707±17,560 (15,846)	24,105±22,516 (17,467)	10,554±10,188 (7,271)	10,625±9,578 (8,117)	P<0.0001	IL-8 v IL-6 P<0.001	
IL-6	1,066±2,332 (379)	860±1,465 (356)	456±805 (183)	334±327 (232)		IL-6 v TNF $\alpha$ P<0.001	
TNF- $\alpha$	520±1,387 (97)	287±395 (158)	120±165 (67)	169±535 (81)			TNF $\alpha$ v IL-1 $\beta$ ns
IL-1 $\beta$	243±557 (69)	129±185 (73)	107±224 (34)	53±52 (33)			
Comparisons between patients & controls					Mann-Whitney		
IL-8	*		*		P<0.0001		
		*		*	P<0.0001		
IL-6	*		*		P=0.001		
		*		*	P=0.0104		
TNF- $\alpha$	*		*		P=0.0127		
		*		*	P=0.0027		
IL-1 $\beta$	*		*		P=0.0029		
		*		*	P=0.0014		

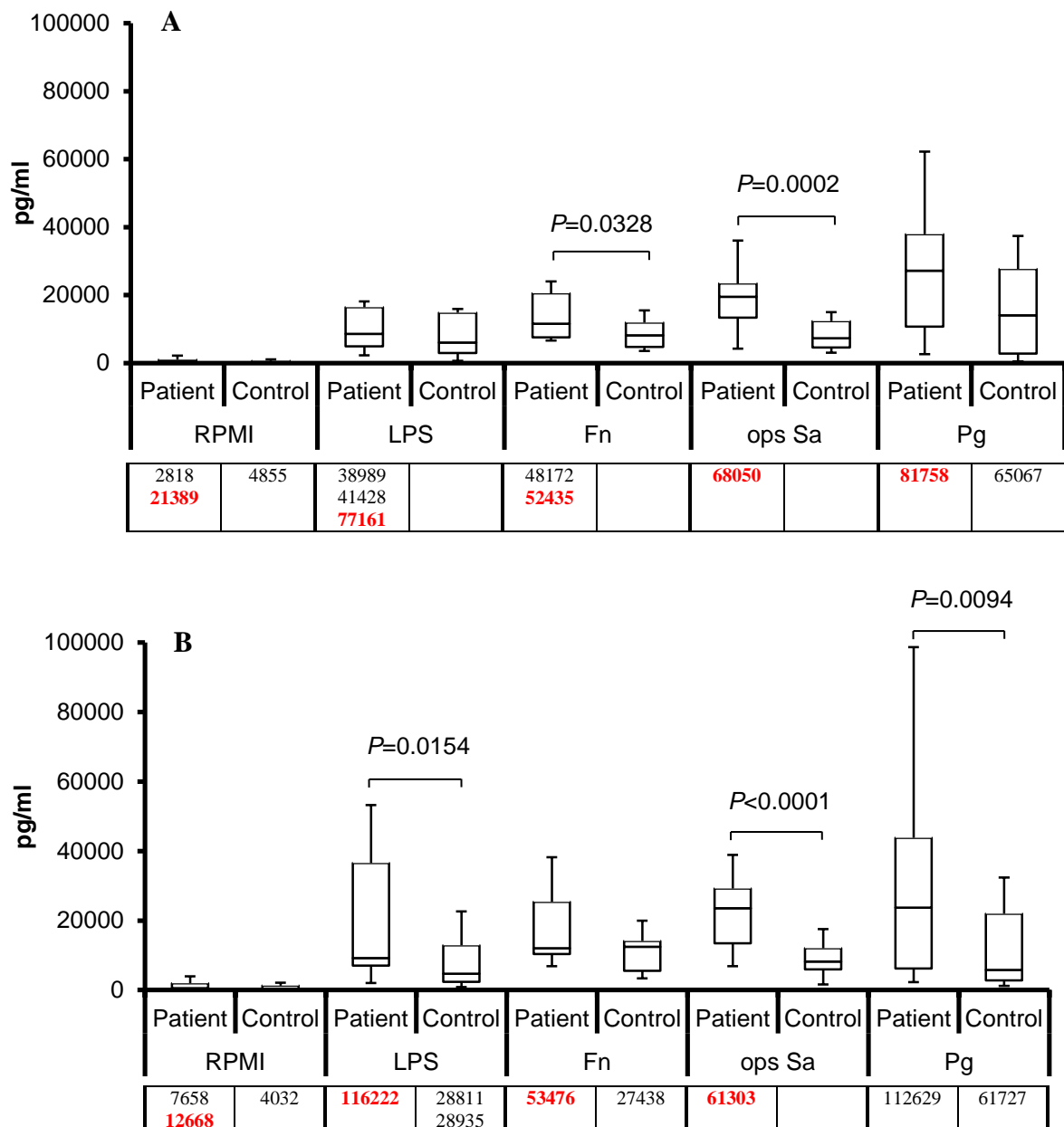
**Table 4.2. Summary of the differences in stimulated IL-8, IL-6, TNF- $\alpha$  and IL-1 $\beta$  release (pg/ml) from patient and control neutrophils at baseline and review in the presence of *E. coli* LPS (5 $\mu$ g/ml), *F. nucleatum* (MOI 100:1), IgG-opsonised *S. aureus* (MOI 300:1) and *P. gingivalis* (MOI 100:1). Data presented as mean±SD (median) pg/ml for all stimuli combined. *P*-values calculated using Kruskal-Wallis test followed by Dunn's multiple comparison's test (differences between cytokine levels for patient and control cells including baseline and review visits; ns: not significant. *P*-values apply to all four cytokine comparisons), and Mann-Whitney test (differences between patients & controls comparing highlighted/starred groups).**



#### 4.4.1 Neutrophil IL-8 release

Baseline IL-8 release from patient and control neutrophils is shown in Figure 4.2A. Overall, patient neutrophils released significantly greater amounts of IL-8 compared to controls when comparing the combined data for all stimuli (Table 4.3;  $P < 0.0001$ ). When examining data for individual stimuli, patient neutrophils released significantly more IL-8 compared to controls following Fc $\gamma$ R (median 19,538 & 7,271pg/ml;  $P = 0.0002$ ) and *F. nucleatum* stimulation only (median 11,575 & 8,189pg/ml;  $P = 0.0328$ ; Figure 4.2A).

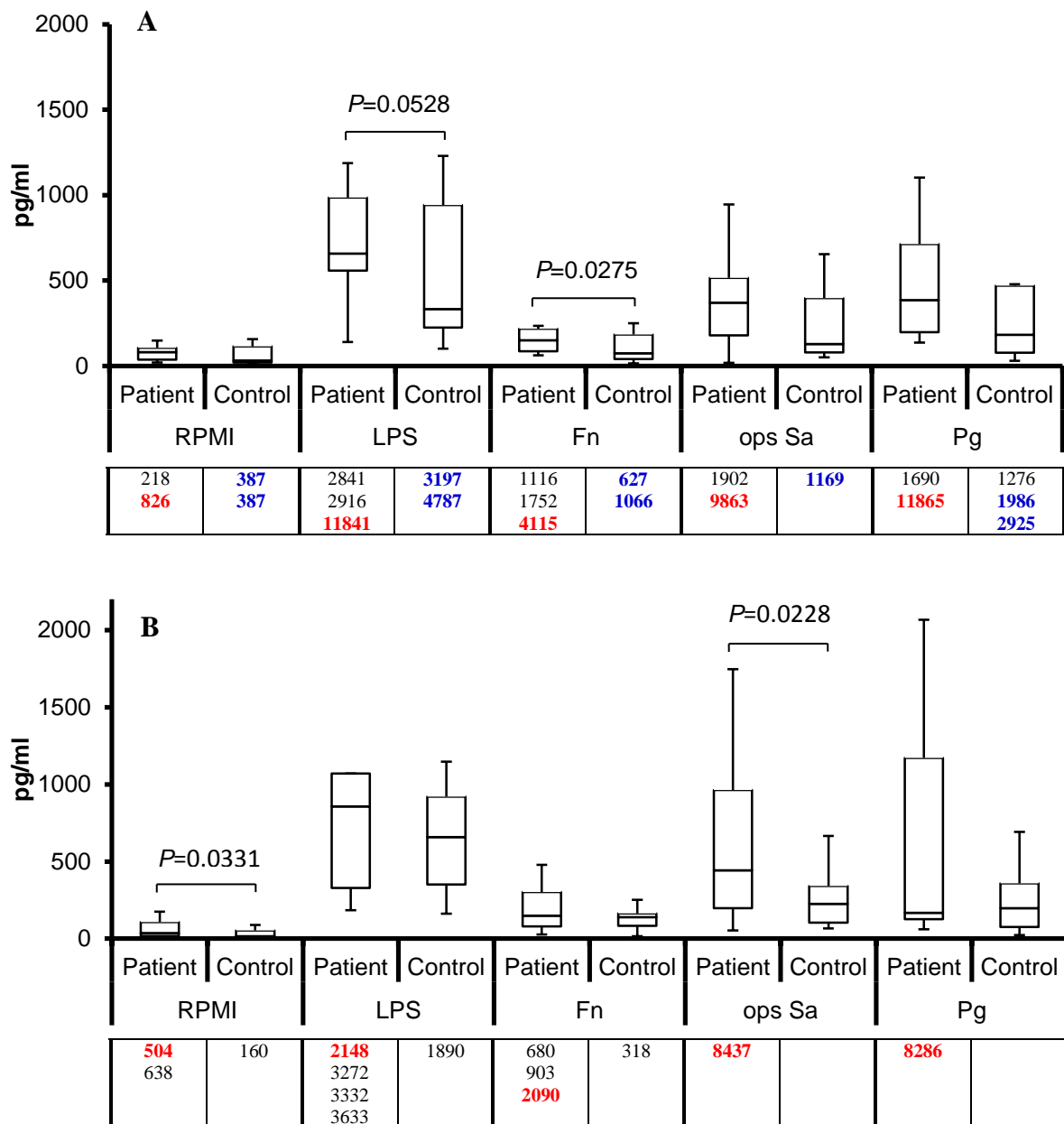
Release of IL-8 from patient and control neutrophils at review is shown in Figure 4.2B. In agreement with the baseline data, patient neutrophils released significantly greater amounts of IL-8 than controls when comparing the combined data for all stimuli (Table 4.3;  $P < 0.0001$ ). In terms of the effect of individual stimuli, patient neutrophils released significantly more IL-8 compared to controls following Fc $\gamma$ R (median 23,540 & 8,171pg/ml;  $P < 0.0001$ ), *P. gingivalis* (median 23,727 & 5,796pg/ml;  $P = 0.0094$ ) and *E. coli* LPS stimulation (median 9,211 & 4,686pg/ml;  $P = 0.0154$ ) but not after stimulation with *F. nucleatum* (Figure 4.2B).



**Figure 4.2. Stimulated IL-8 production by neutrophils (A) baseline, pre-treatment (n=18) and (B) review, post-treatment (n=18) after 18 hours culture in the absence (RPMI control) or presence of *E. coli* LPS (5µg/ml), *F. nucleatum* (MOI 100:1), IgG-opsonised *S. aureus* (MOI 300:1) and *P. gingivalis* (MOI 100:1).** Box and whisker plots showing median, interquartile range, maximum and minimum. Values for outliers (calculated as >1.5xIQR) are tabulated as follows. (A) patient volunteer no. 13 gave consistently extreme IL-8 levels and are shown in red; (B) patient volunteer no. 6 gave consistently extreme IL-8 levels and are shown in red. *P*-values calculated using two-tailed Mann-Whitney tests.

#### 4.4.2 Neutrophil IL-6 release

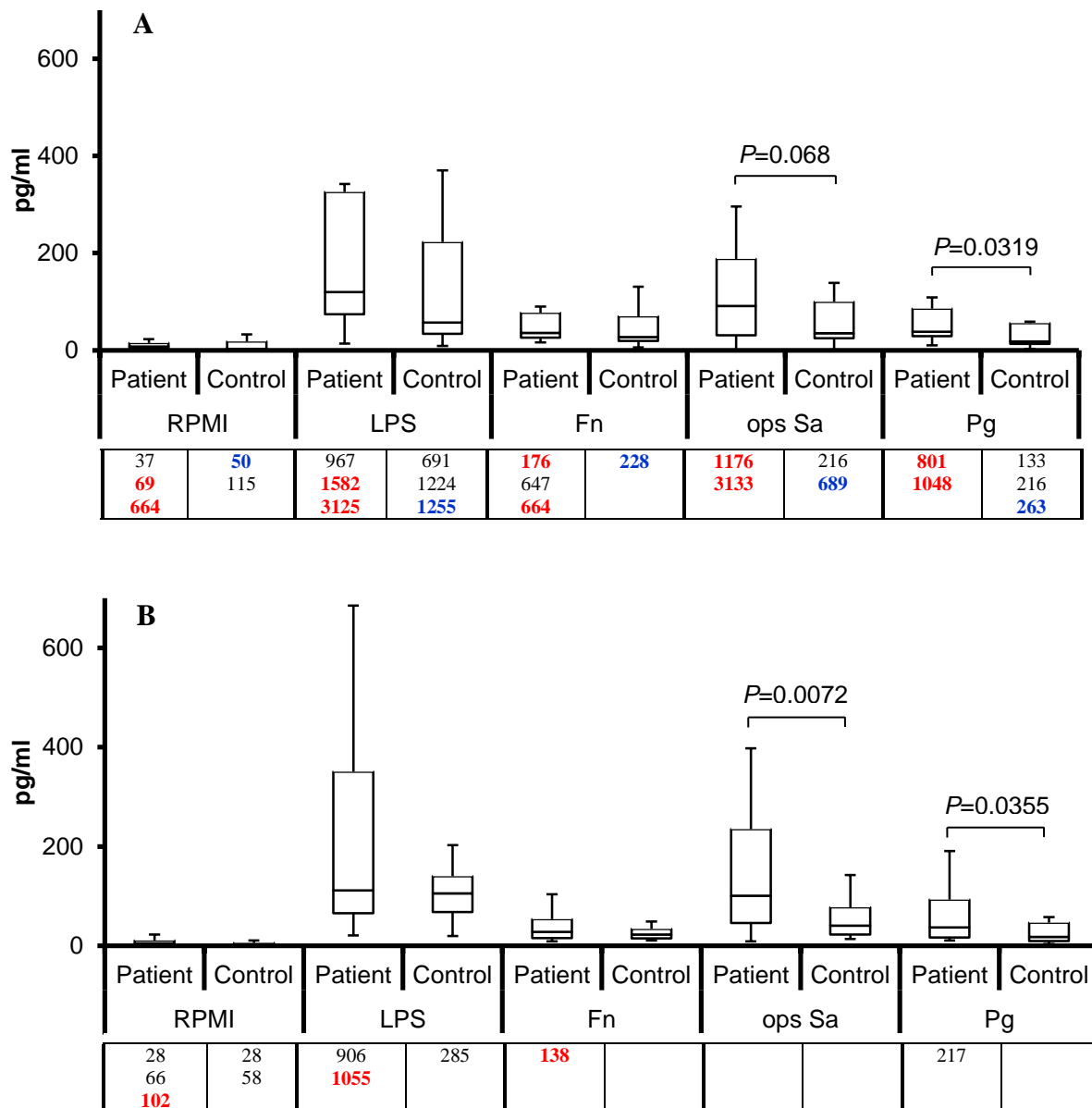
Baseline IL-6 release from patient and control neutrophils is shown in Figure 4.3A. Patient neutrophils released greater amounts of IL-6 compared to controls that was significant when comparing the combined IL-6 concentrations (Table 4.3;  $P=0.001$ ). Similarly, patient neutrophils released more IL-6 compared to controls in the presence of *F. nucleatum* (median 150 & 74pg/ml;  $P=0.0275$ ) and following stimulation with *E. coli* LPS (median 658 & 333pg/ml;  $P=0.0528$ ). Release of IL-6 from patient and control neutrophils at review is shown in Figure 4.3B. In agreement with the baseline data, patient neutrophils released more IL-6 than controls at review, which was significant when comparing the combined IL-6 concentrations (Table 4.3;  $P=0.0104$ ). In addition, patient neutrophils released significantly more IL-6 compared to controls following Fc $\gamma$ R stimulation at review (median 443 & 225pg/ml;  $P=0.0228$ ) but not after stimulation with *E.coli* LPS or *F. nucleatum*.



**Figure 4.3. Stimulated IL-6 production by neutrophils (A) baseline, pre-treatment (n=17) and (B) review, post-treatment (n=17) after 18 hours culture in the absence (RPMI control) or presence of *E. coli* LPS (5µg/ml), *F. nucleatum* (MOI 100:1), IgG-opsonised *S. aureus* (MOI 300:1) and *P. gingivalis* (MOI 100:1).** Box and whisker plots showing median, interquartile range, maximum and minimum. Values for outliers (calculated as  $>1.5 \times \text{IQR}$ ) are tabulated as follows. (A) control volunteer no.'s 10 & 12 gave consistently high IL-6 levels and are shown in blue. Extreme values in red were obtained from neutrophil cultures from patient volunteer no. 13; (B) patient volunteer no. 6 gave consistently high IL-6 levels and are shown in red. *P*-values calculated using two-tailed Mann-Whitney tests.

#### **4.4.3 Neutrophil IL-1 $\beta$ release**

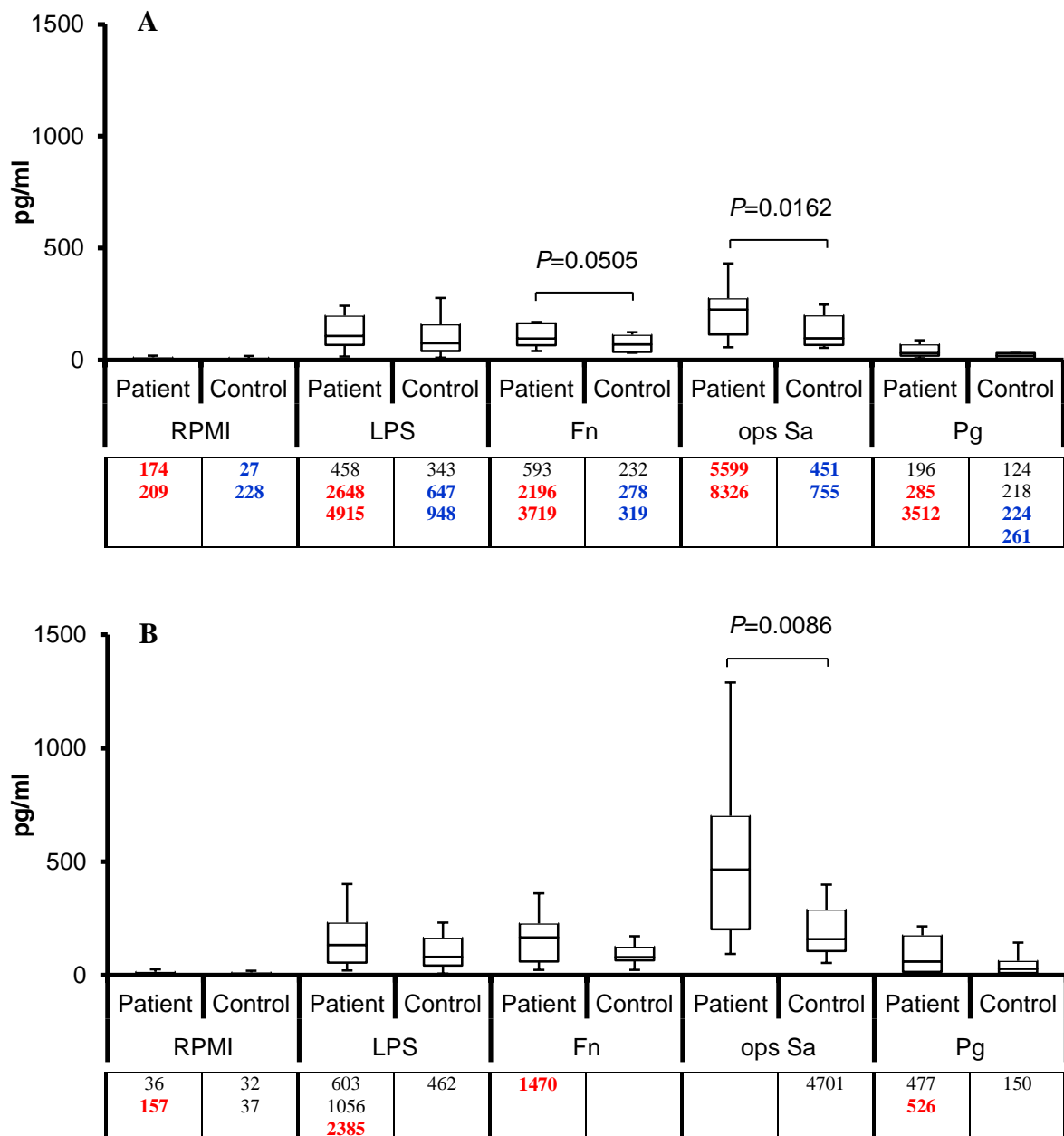
Baseline IL-1 $\beta$  release from patient and control neutrophils is shown in Figure 4.4A. Neutrophils from periodontitis patients released significantly greater amounts of IL-1 $\beta$  compared to controls when comparing the combined IL-1 $\beta$  concentrations (Table 4.3;  $P=0.0029$ ). Patient neutrophils similarly released more IL-1 $\beta$  compared to controls following stimulation with *P. gingivalis* (median 38 & 18pg/ml;  $P=0.0319$ ) and also Fc $\gamma$ R stimulation (median 91 & 35pg/ml;  $P=0.068$ ). Review IL-1 $\beta$  release from patient and control neutrophils is shown in Figure 4.4B. In agreement with the baseline data, patient neutrophils released greater amounts of IL-1 $\beta$  than controls at review, which was significant when comparing the combined IL-1 $\beta$  concentrations (Table 4.3;  $P<0.0014$ ). Patient neutrophils continued to release significantly more IL-1 $\beta$  compared to control cells following Fc $\gamma$ R (median 101 & 41pg/ml;  $P<0.0072$ ) and *P. gingivalis* stimulation (median 37 & 18pg/ml;  $P=0.0355$ ).



**Figure 4.4. Stimulated IL-1 $\beta$  production by neutrophils (A) baseline, pre-treatment (n=19) and (B) review, post-treatment (n=19) after 18 hours culture in the absence (RPMI control) or presence of *E. coli* LPS (5 $\mu$ g/ml), *F. nucleatum* (MOI 100:1), IgG-opsonised *S. aureus* (MOI 300:1) and *P. gingivalis* (MOI 100:1).** Box and whisker plots showing median, interquartile range, maximum and minimum. Values for outliers (calculated as  $>1.5 \times \text{IQR}$ ) are tabulated as follows. (A) control volunteer no. 10 gave consistently high IL-1 $\beta$  levels and are shown in blue. Extreme values in red were obtained from neutrophil cultures from patient volunteer no.'s 6 & 13; (B) patient volunteer no. 6 gave consistently extreme IL-1 $\beta$  levels and are shown in red. *P*-values calculated using two-tailed Mann-Whitney tests.

#### 4.4.4 Neutrophil TNF- $\alpha$ release

Baseline TNF- $\alpha$  release from patient and control neutrophils is shown in Figure 4.5A. Periodontitis patient neutrophils released significantly more TNF- $\alpha$  compared to controls when comparing the combined TNF- $\alpha$  concentrations (Table 4.3;  $P=0.0127$ ). Patient neutrophils released more TNF- $\alpha$  compared to controls following Fc $\gamma$ R (median 225 & 98pg/ml;  $P=0.0162$ ) and also *F. nucleatum* stimulation (median 96 & 70pg/ml;  $P=0.0505$ ). Review TNF- $\alpha$  release from patient and control neutrophils is shown in Figure 4.5B. In agreement with the baseline data, periodontitis neutrophils released greater amounts of TNF- $\alpha$  than controls at review which was significant when comparing the combined TNF- $\alpha$  concentrations (Table 4.3;  $P<0.0027$ ). Patient neutrophils continued to release significantly more TNF- $\alpha$  compared to control cells following Fc $\gamma$ R stimulation (median 465 & 159pg/ml;  $P=0.0086$ ) but not following TLR stimulation with *F. nucleatum*.



**Figure 4.5. Stimulated TNF- $\alpha$  production by neutrophils (A) baseline, pre-treatment (n=19) and (B) review, post-treatment (n=19) after 18 hours culture in the absence (RPMI control) or presence of *E. coli* LPS (5 $\mu$ g/ml), *F. nucleatum* (MOI 100:1), IgG-opsonised *S. aureus* (MOI 300:1) and *P. gingivalis* (MOI 100:1).** Box and whisker plots showing median, interquartile range, maximum and minimum. Values for outliers (calculated as  $>1.5 \times \text{IQR}$ ) are tabulated as follows. (A) control volunteer no.'s 5 & 10 gave consistently high TNF- $\alpha$  levels and are shown in blue Extreme values in red were obtained from neutrophil cultures from patient volunteer no.'s 6 & 13; (B) patient volunteer no. 6 gave consistently extreme TNF- $\alpha$  levels and are shown in red. *P*-values calculated using two-tailed Mann-Whitney tests.



#### **4.5 SUMMARY OF DIFFERENCES IN CYTOKINE RELEASE BY PATIENT & CONTROL NEUTROPHILS**

A summary of the differences in baseline and review IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  release between patient and control neutrophils in the presence and absence of individual stimuli is shown in Table 4.4. Cytokine release from control cells was always similar to, or less than, that released from patient cells. There were no differences in unstimulated cytokine release by patient and control neutrophils except for the greater release of IL-6 by patient cells at review. By contrast, at baseline and review, various cytokines were produced in higher amounts by patient neutrophils after stimulation. Patient neutrophils released greater amounts of IL-6, IL-8 and TNF- $\alpha$  than controls in the presence of *F. nucleatum* at baseline ( $P \leq 0.05$ ), with no significant differences at review. Increased Fc $\gamma$ R stimulated neutrophil cytokine release was only significant for IL-8 and TNF- $\alpha$  at baseline, however Fc $\gamma$ R stimulated hyper-reactivity was a consistent feature of periodontitis neutrophils for all cytokines at review ( $P \leq 0.0228$ ). In addition, *P. gingivalis* stimulated IL-1 $\beta$  hyper-reactivity was a consistent feature of periodontitis neutrophils at baseline and review ( $P \leq 0.0355$ ).

Cytokine	Stimulus	Difference in cytokine release between patient & control cells	
		Baseline	Review
IL-6 (n=17)	RPMI control	↔	↑ (P=0.0331)
	<i>E. coli</i> LPS	↑ (P=0.0582)	↔
	<i>F. nucleatum</i>	↑ (P=0.0275)	↔
	ops <i>S. aureus</i>	↔	↑ (P=0.0228)
	<i>P. gingivalis</i>	↔	↔
IL-8 (n=18)	RPMI control	↔	↔
	<i>E. coli</i> LPS	↔	↑ (P=0.0154)
	<i>F. nucleatum</i>	↑ (P=0.0328)	↔
	ops <i>S. aureus</i>	↑ (P=0.0002)	↑ (P<0.0001)
	<i>P. gingivalis</i>	↔	↑ (P=0.0094)
IL-1β (n=19)	RPMI control	↔	↔
	<i>E. coli</i> LPS	↔	↔
	<i>F. nucleatum</i>	↔	↔
	ops <i>S. aureus</i>	↔	↑ (P=0.0072)
	<i>P. gingivalis</i>	↑ (P=0.0319)	↑ (P=0.0355)
TNF-α (n=19)	RPMI control	↔	↔
	<i>E. coli</i> LPS	↔	↔
	<i>F. nucleatum</i>	↑ (P=0.0505)	↔
	ops <i>S. aureus</i>	↑ (P=0.0162)	↑ (P=0.0086)
	<i>P. gingivalis</i>	↔	↔

**Table 4.3. Summary of the difference in cytokine release between patient and control neutrophils at baseline and review after 18 hours culture in the absence (RPMI control) or presence of *E. coli* LPS (5µg/ml), *F. nucleatum* (MOI 100:1), IgG-opsonised *S. aureus* (MOI 300:1) and *P. gingivalis* (MOI 100:1). ↑ signifies patient>control and ↔ signifies no difference. *P*-values calculated using two-tailed Mann-Whitney tests.**

#### **4.6 CHANGE IN REVIEW COMPARED WITH BASELINE CYTOKINE RELEASE BY PATIENT & CONTROL NEUTROPHILS**

The changes in neutrophil IL-6, IL-8, IL-1β and TNF-α release between baseline and review sampling visits are summarised in Table 4.5. Control neutrophils released less IL-6 at review compared to baseline in the absence of stimulation (RPMI control; *P*=0.029), which may partly explain the greater IL-6 release from patient compared to control neutrophils found at review (*P*=0.0331; Figure 4.3; Table 4.4). The remaining changes in cytokine release from

neutrophils between sampling visits did not influence any of the other differences detected in cytokine release from patient and control neutrophils (Figures 4.2, 4.4, 4.5; Table 4.4).

Cytokine	Stimulus	Changes in cytokine release between baseline and review	
		Patient	Control
IL-6 (n=17)	RPMI control	↔	↓ ( $P = 0.029$ )
	<i>E. coli</i> LPS	↔	↔
	<i>F. nucleatum</i>	↔	↔
	ops <i>S. aureus</i>	↔	↔
	<i>P. gingivalis</i>	↔	↔
IL-8 (n=18)	RPMI control	↑ ( $P = 0.0448$ )	↔
	<i>E. coli</i> LPS	↔	↔
	<i>F. nucleatum</i>	↔	↔
	ops <i>S. aureus</i>	↔	↔
	<i>P. gingivalis</i>	↔	↔
IL-1 $\beta$ (n=19)	RPMI control	↔	↔
	<i>E. coli</i> LPS	↔	↔
	<i>F. nucleatum</i>	↔	↓ ( $P = 0.029$ )
	ops <i>S. aureus</i>	↔	↔
	<i>P. gingivalis</i>	↔	↔
TNF- $\alpha$ (n=19)	RPMI control	↔	↔
	<i>E. coli</i> LPS	↔	↔
	<i>F. nucleatum</i>	↔	↔
	ops <i>S. aureus</i>	↓ ( $P = 0.0079$ )	↔
	<i>P. gingivalis</i>	↑ ( $P = 0.0174$ )	↔

**Table 4.4. Summary of the changes in cytokine release between baseline and review for patient and control neutrophils after 18 hours culture in the absence (RPMI control) or presence of *E. coli* LPS (5 $\mu$ g/ml), *F. nucleatum* (MOI 100:1), IgG-opsonised *S. aureus* (MOI 300:1) and *P. gingivalis* (MOI 100:1). ↑ review > baseline, ↓ review < baseline & ↔ no change. *P*-values calculated using two-tailed Wilcoxon signed-rank test.**

## 4.7 DISCUSSION

The cytokine data presented in this chapter demonstrates, for the first time, that peripheral blood neutrophils from untreated patients with chronic periodontitis exhibit hyper-reactive IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  release compared with healthy controls. The current work is also the first longitudinal intervention study analysing the impact of therapeutic reductions in periodontal infection and inflammation on the ability of peripheral blood neutrophils to generate cytokines in the presence and absence of periodontally relevant bacteria. This study reports *F. nucleatum* stimulated hyper-reactivity with regards to IL-6, IL-8 and TNF- $\alpha$  release when comparing patient and control volunteers at baseline that was not detected at review. The data also demonstrate Fc $\gamma$ R-stimulated IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  and *P. gingivalis*-stimulated IL-1 $\beta$  hyper-reactivity when comparing patient and control volunteers at baseline that was maintained at review. Together, these data are consistent with current literature highlighting a dysregulated neutrophil phenotype in untreated chronic periodontitis, and this is biologically significant given that cytokines regulate many different aspects of the inflammatory-immune response, including neutrophil ROS and NET release as well as chemotactic potential.

Monocytes and lymphocytes were originally thought to be the predominant source of pro-inflammatory cytokines in inflammatory lesions. However, the data presented in this thesis confirms that neutrophils are similarly able to release IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  and are consistent with current literature (Cassatella *et al.* 1997, 1999). This study also confirms previous studies showing that neutrophils release significantly greater amounts of IL-8 and IL-6 compared to IL-1 $\beta$  and TNF- $\alpha$  (Altstaedt *et al.* 1996, Retini *et al.* 1996). This finding may relate to the ability of IL-1 $\beta$  and TNF- $\alpha$  to act synergistically to induce expression of IL-8 and IL-6 (Kent *et al.* 1998, Hattar *et al.* 2006). Given the relatively high levels of IL-8 production detected in this thesis, it is not surprising that neutrophils were initially thought to produce only IL-8 (Bazzoni *et al.* 1991, Cassatella *et al.* 1992, 1993, Arnold *et al.* 1994, Wei *et al.* 1994, Altstaedt *et al.* 1996). Significantly, given the large amounts of IL-8 released, it is relevant that IL-8 is not only an important chemo-attractant (Hammond *et al.* 1995), but also has the ability to prime neutrophils for ROS release (Gainet *et al.* 1998, Van Dervort *et al.* 1998, Guichard C *et al.* 2005, Dias *et al.* 2011, Fredriksson 2012) and NET release (Brinkman

*et al.* 2004). Therefore, neutrophil-derived IL-8 is likely to play a major role in regulating key inflammatory-immune responses generally and in periodontitis in particular.

Although mean and median levels of unstimulated cytokine release were highest for patient cells, the difference, compared to control cell levels, was not significant for either the combined or individual cytokine data, except for IL-6 at review. This latter result appeared to be the result of a decrease in control neutrophil IL-6 release at review compared to baseline, despite no known intervention between the sampling visits. While further studies on larger numbers are required to determine whether the greater release of cytokines by patient cells is statistically significant, it is interesting to speculate that even small differences may be biologically important in the pathogenesis of chronic periodontitis, given the large numbers of systemic and locally recruited neutrophils within the periodontium.

To date, there are only 4 published studies investigating peripheral blood neutrophil cytokine release in chronic periodontitis (Galbraith *et al.* 1997, Figueredo *et al.* 2000, Fredriksson *et al.* 2002, Restaino *et al.* 2007). Whilst Figueredo *et al.* has previously reported greater unstimulated neutrophil IL-1 $\beta$  release from patient compared to control neutrophils, the study used flow cytometric analyses of fixed and permeabilised neutrophils which, as such, cannot measure active IL-1 $\beta$  release (Figueredo *et al.* 2000). The data presented in this chapter are in agreement with those of Galbraith *et al.* in that there were no significant differences in IL-1 $\beta$  release from patient and control neutrophils in the absence of stimulation (Galbraith *et al.* 1997). However, this study detected TNF- $\alpha$  production by both unstimulated and stimulated neutrophils whereas Galbraith and co-workers failed to detect TNF- $\alpha$  in the presence or absence of priming with GM-CSF, possibly relating to a shorter 5 hour neutrophil cell culture time (Galbraith *et al.* 1997). The lack of significant difference in unstimulated IL-8 release from patient and control neutrophils in this chapter also contrasts with a study suggesting lower IL-8 release from periodontitis neutrophils (Restaino *et al.* 2007). This discrepancy in the data is likely to result from differences in neutrophil isolation method used, with inadvertent cell activation of neutrophils occurring in the study of Restaino and co-workers (2007).

There is a relative lack of published studies investigating peripheral blood neutrophil cytokine release in human disease. The data presented in this chapter are in agreement with studies demonstrating no difference in unstimulated IL-8 release from neutrophils isolated from patients diagnosed with obstructive sleep apnea (Guasti *et al.* 2011) or chronic obstructive pulmonary disease (Blidberg *et al.* 2012) compared to those from healthy controls. By contrast, greater unstimulated IL-8, IL-1 $\beta$  and TNF- $\alpha$  release has been reported for neutrophils from patients with type-2 diabetes compared to those from healthy control volunteers using a study design similar to that used in the current work (Hatanaka *et al.* 2006). However, the cytokine levels detected were much larger than those in this chapter which may be representative of the increased inflammatory burden associated with type-2 diabetes compared to chronic periodontitis.

All stimuli resulted in significantly increased neutrophil release of all cytokines except for *F. nucleatum*, which failed to significantly stimulate IL-6 release. Whilst *F. nucleatum* stimulated neutrophils have been shown to release IL-8, IL-1 $\beta$ , TNF- $\alpha$  (Yoshimura *et al.* 1997, Sheikhi *et al.* 2000), both of these studies did not investigate neutrophil IL-6 release. In addition, there are no known published studies investigating neutrophil IL-6 release in the presence of the periodontal pathogen *F. nucleatum*, despite published literature demonstrating *F. nucleatum* stimulated IL-6 release from monocytes and gingival fibroblasts (Rossano *et al.* 1993). The data in this thesis therefore provides important and unexpected novel data in that *F. nucleatum* does not significantly stimulate neutrophil IL-6 release.

Although cytokine hyperactivity (increased cytokine release in the absence of stimulation) was not detected, greater stimulated IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  release from patient compared to control neutrophils was a consistent feature when comparing data for all stimuli combined, suggesting that patient cells were hyper-reactive in terms of cytokine production. Neutrophil cytokine hyper-reactivity in the absence of hyperactivity contrasts with the peripheral blood neutrophil ROS hyperactivity and hyper-reactivity characteristic of untreated chronic periodontitis (reviewed by Chapple & Matthews 2007; section 5.3).

In terms of responses to individual stimuli, patient neutrophils released greater amounts of IL-6, IL-8 and TNF- $\alpha$  in the presence of *F. nucleatum*, greater Fc $\gamma$ R-stimulated IL-8 and TNF- $\alpha$ ,

and greater *P. gingivalis*-stimulated IL-1 $\beta$  compared to controls. The Fc $\gamma$ R-stimulated IL-8 and TNF- $\alpha$  hyper-reactivity presented in this thesis contrasts with the lack of significant differences previously reported for periodontitis and control neutrophils (Fredriksson *et al.* 2002). However Fredriksson *et al.* utilised a 5 hour culture time and detected only low levels of IL-8 (range 16-64pg/ml) and TNF- $\alpha$  (range 13-248pg/ml) compared to those presented in this chapter (ranges 1,640-68,050pg/ml and 54-8,326pg/ml for IL-8 and TNF- $\alpha$  respectively) using an 18 hour culture period. The kinetics of neutrophil cytokine generation have been described and have shown that the majority of stimulated neutrophil IL-1 $\beta$ , IL-8, IL-6, TNF- $\alpha$  release occurs at 16-18 hours (Cassatella *et al.* 1993, Hattar *et al.* 2006, Retini *et al.* 1996), with neutrophil IL-8, IL-1 $\beta$  and TNF- $\alpha$  release gradually decreasing after 16 hours (Hattar *et al.* 2006). The lack of significant difference in *E. coli* LPS and *P. gingivalis* stimulated IL-8 release from patient and control neutrophils presented in this chapter contrasts with a study reporting lower *E. coli* LPS and *P. gingivalis* stimulated IL-8 release from periodontitis neutrophils (Restaino *et al.* 2007). However the discrepancies in both unstimulated and stimulated IL-8 release with Restaino *et al.* may be related to differences in neutrophil isolation method including the potential for inadvertent cell activation. This is because extremely high amounts of IL-8 (~800ng/ml) were detected from control neutrophils along with a lack of observable stimulation. Taken together this suggests cell activation during isolation therefore causing heightened IL-8 release compared to relatively inactivated neutrophils isolated from periodontitis patients (Restaino *et al.* 2007).

With regards to *in-vitro* methodology, it is also important to confirm neutrophil viability following culture in order to distinguish between active cytokine release rather than passive or low-level release due to cell necrosis or apoptosis. Such details are not included in the published neutrophil cytokine studies in chronic periodontitis. Whilst this was confirmed in this study, with neutrophil viability confirmed following 18 hour culture, there was a small but statistically significant decrease in neutrophil viability in the presence of IgG-opsonised *S. aureus* compared with the other culture conditions, although this did not appear to affect the amount of IL-8, IL-6, IL-1 $\beta$  or TNF- $\alpha$  released compared to the other stimuli. The small but significant reduction in neutrophil viability in the presence of IgG-opsonised *S. aureus* may be the result of increased phagocytosis due to bacterial opsonisation, as well as the multiplicity of infection (MOI) used compared to the other, non-opsonised, bacterial stimuli,

namely MOI 300:1 compared with an MOI of 100:1 when including unopsonised periodontal pathogens. The MOI's were chosen as indicated (section 2.2.12) namely in agreement with similar studies investigating neutrophil responses to unopsonised and opsonised bacteria to elicit measurable neutrophil responses whilst maintaining neutrophil viability. In addition, opsonised *S. aureus* neutrophil stimulation may additionally result from lipoteichoic acid (LTA) binding via the outer cell membrane of Gram-positive bacteria (Hatter *et al.* 2006). It is therefore also possible that neutrophil viability in the presence of IgG-opsonised *S. aureus* is negatively influenced by multiple receptor binding following bacterial opsonisation. Furthermore, opsonised *S. aureus* stimulation leads to increased NETosis (personal communication P.White August 2014), which may also negatively influence neutrophil viability.

The effect of periodontal treatment on neutrophil cytokine release was also determined. The data presented in this thesis was unable to demonstrate that periodontal treatment significantly reduced neutrophil cytokine release when comparing baseline and review visits for the patient volunteers. Therefore, neutrophil cytokine hyper-reactivity observed at baseline which was no longer present at review cannot reliably be attributed solely to the effect of periodontal treatment itself. The only exception was the significantly reduced neutrophil FcγR-stimulated TNFα release for the patient volunteers between baseline and review visits. Interestingly, *F. nucleatum*-stimulated IL-6, IL-8 and TNF-α hyper-reactivity of patient neutrophils at baseline was not observed at review following periodontal treatment. However, as previously discussed, careful data interpretation is required given that patient neutrophil cytokine release did not significantly reduce following therapy for these parameters. However, it is interesting to speculate that the non-significant differences between patient and control volunteers at review may be biologically significant and could potentially suggest a benefit of periodontal treatment regarding systemic host inflammatory-immune responses. In contrast, consistently heightened FcγR-stimulated cytokine release from patient compared to control neutrophils was observed both at baseline and review, despite the aforementioned significant reduction in neutrophil FcγR-stimulated TNFα release for the patient volunteers in between the sampling visits. Whilst this may suggest that FcγR neutrophil cytokine hyper-reactivity is a constitutive feature in chronic periodontitis, it contrasts with data from our research group that have shown that FcγR-stimulated ROS hyper-reactivity is corrected following treatment (Matthews

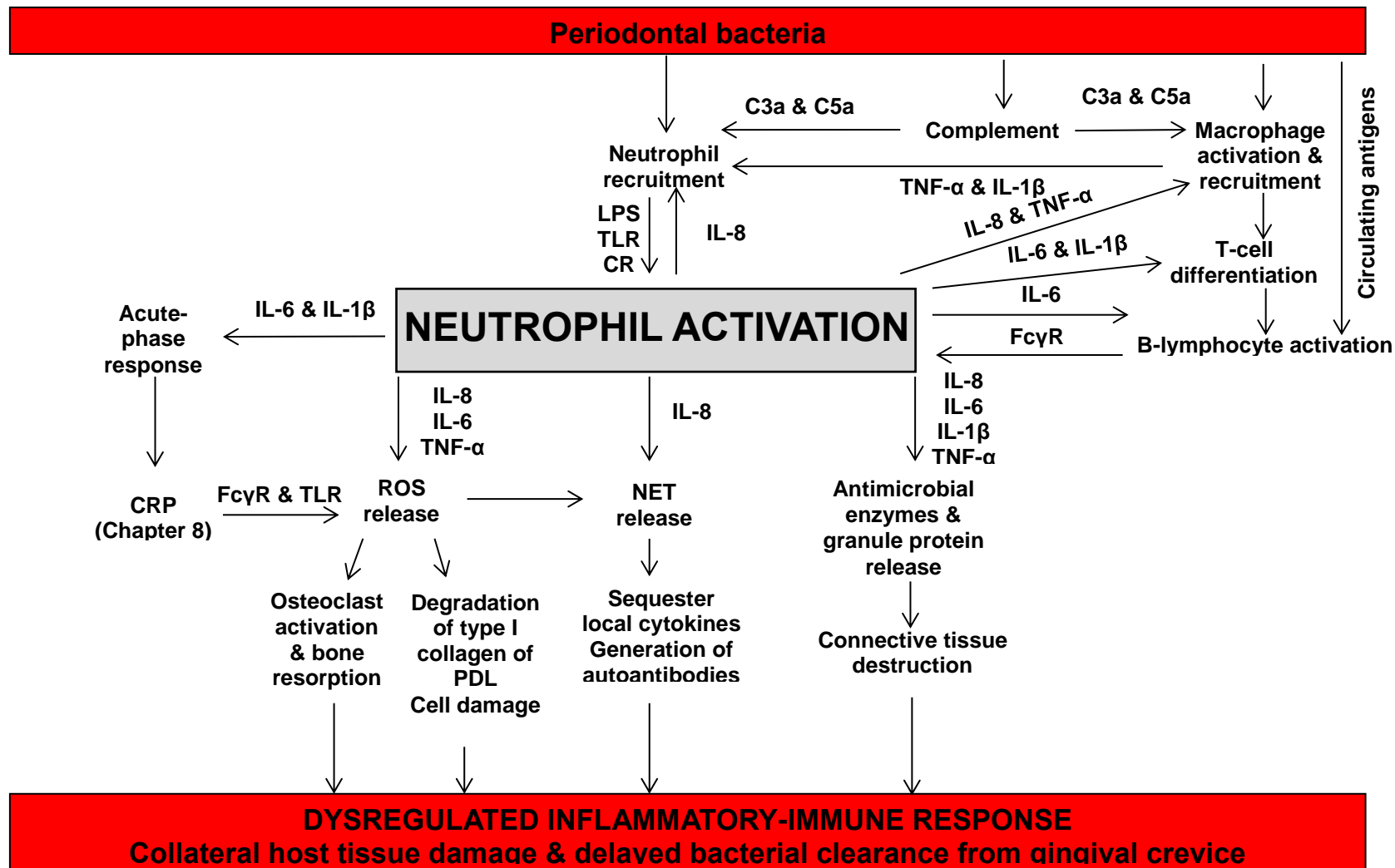


*et al.* 2007). Greater *P. gingivalis*-stimulated IL-1 $\beta$  release from patient compared to control neutrophils was similarly a consistent finding at both baseline and post-treatment review. *Porphyromonas gingivalis* is strongly associated with chronic periodontitis pathogenesis (Socransky *et al.* 1998), and *P. gingivalis* LPS is structurally distinct from other sources of LPS (Ogawa 1993) and induces cytokine production via different signalling mechanisms (Diya *et al.* 2008). *Porphyromonas gingivalis* LPS is heterogenous, with some *P. gingivalis* LPS molecules binding to TLR-2 and others to TLR-4 and, possibly, also receptor antagonists (Hajishengallis *et al.* 2002, Darveau *et al.* 2004). Furthermore, recent evidence reveals the complexity of IL-1 $\beta$  secretion with two signals required, the first being a TLR-induced transcription of pro-IL-1 $\beta$ , for example binding of LPS to TLR-4, and the second being an additional signal (e.g. extracellular ATP) which results in assembly of an inflammasome, activation of caspase-1 prior to the secretion of mature IL-1 $\beta$  (Latz 2010). Taken together, a constitutive defect in TLR receptor or downstream signalling relating to IL-1 $\beta$  may explain the persistent hyper-reactive *P. gingivalis* stimulated IL-1 $\beta$  release from patient compared to control neutrophils presented in this chapter.

Dysregulated neutrophil cytokine release, namely cytokine hyper-reactivity to periodontal pathogens and Fc $\gamma$ R activation, will have a number of different consequences on the inflammatory-immune response in relation to the pathogenesis of chronic periodontitis as summarised in Figure 4.6. Greater neutrophil IL-8, IL-6 and TNF- $\alpha$  release will result in neutrophil priming for greater ROS release (Guichard *et al.* 2005, Chen *et al.* 2006) which will be further modified by CRP (Chapter 8) following IL-6 and IL-1 $\beta$  activation of the acute-phase response (Heinrich *et al.* 1990, Gabay *et al.* 2001). Whilst a reduction in neutrophil ROS release will result in impaired antimicrobial capability resulting in delayed bacterial clearance from the gingival crevice, greater extracellular ROS release may cause collateral host tissue damage as a result of osteoclast activation causing bone resorption (Lee *et al.* 2005) in addition to degradation of type I collagen of the periodontal ligament (Henrotin *et al.* 2003). Neutrophil ROS and IL-8 release will also result in NET formation (Brinkmann *et al.* 2004, Palmer *et al.* 2012) which, if excessive, may result in the generation of autoantibodies (de Pablo *et al.* 2014) along with impairing the inflammatory-immune response by sequestering local cytokine dissemination (Bank & Ansorge 2001). Neutrophil IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  release will also trigger neutrophil antimicrobial enzyme and granule protein

release with the potential to cause destruction of adjacent periodontal connective tissue (Lacy 2006). In addition, there are a number of indirect effects of dysregulated neutrophil cytokine release that will impact on the pathogenesis of chronic periodontitis. For example, neutrophil IL-8 release is likely to affect concurrent neutrophil chemotaxis and recruitment within the periodontal tissues (Hammond *et al.* 1995; Chapter 6). Furthermore, any adaptive immune response will be heightened by IL-8, IL-6 and TNF- $\alpha$  resulting in T-cell differentiation (Kishimoto *et al.* 1989, Ben-Sasson *et al.* 2009) and B-lymphocyte activation which is also directly influenced by IL-6 (Maeda *et al.* 2010). The generation of antibodies and bacterial opsonisation will result in Fc $\gamma$ R-stimulated neutrophil activation thereby further exacerbating the inflammatory-immune response in relation to the persistent Fc $\gamma$ R-stimulated IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  release presented in this chapter and Fc $\gamma$ R-stimulated neutrophil ROS hyper-reactivity in chronic periodontitis (Matthews *et al.* 2007).

Overall the neutrophil cytokine release data presented as part of this thesis demonstrate that neutrophils from untreated periodontitis patients are hyper-reactive with regards to IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  release compared to cells from healthy control individuals. Furthermore consistent Fc $\gamma$ R-stimulated IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  and *P. gingivalis* stimulated IL-1 $\beta$  neutrophil hyper-reactivity appears to be a constitutive and consistent feature of the pathogenesis of chronic periodontitis. Despite therapy having no detectable effect on neutrophil cytokine production in response to *F. nucleatum*, the apparent loss of *F. nucleatum*-stimulated IL-6, IL-8 and TNF- $\alpha$  hyper-reactivity of patient neutrophils at review could potentially highlight a systemic benefit of periodontal therapy that might reduce the risk of co-morbidity associated with a dysregulated systemic inflammatory-immune response.



**Figure 4.6. Overview of neutrophil cytokine release in the pathogenesis of chronic periodontitis.** Abbreviations: LPS, lipopolysaccharide; TLR, toll-like receptor; CR, complement receptor; IL, interleukin; C, complement; TNF, tumour necrosis factor; CRP, C-reactive protein; FcyR, Fcy-receptor; ROS, reactive oxygen species; NET, neutrophil extracellular trap; PDL, periodontal ligament.

# **CHAPTER 5**

## **RESULTS & DISCUSSION**

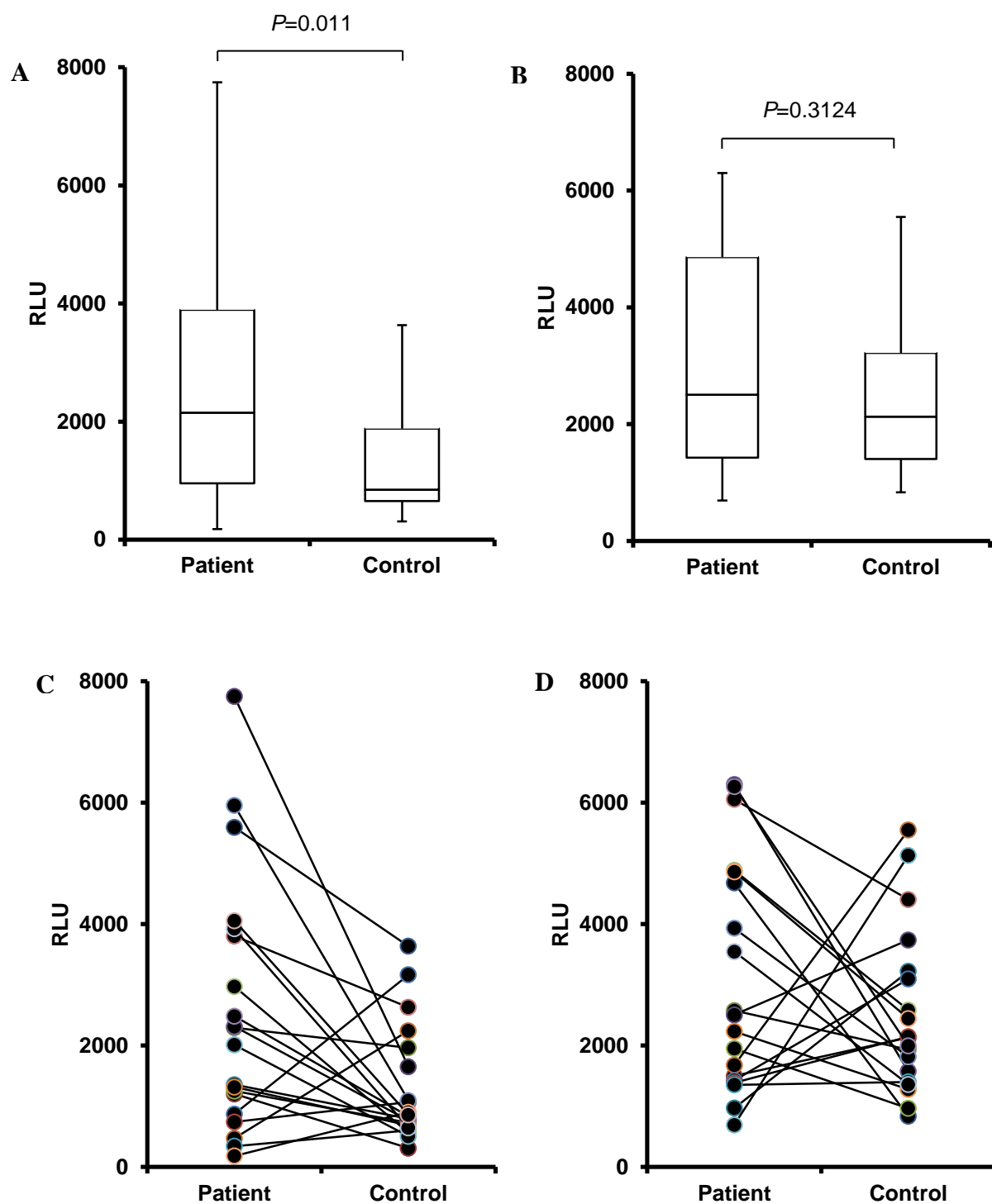
**Case-control longitudinal intervention study:  
Neutrophil superoxide release in chronic periodontitis**

## 5.1 INTRODUCTION

Lucigenin-detectable superoxide ( $O_2^-$ ) release from peripheral blood neutrophils isolated from patients with chronic periodontitis pre- and post-treatment was determined and compared to that of cells from healthy controls. The aim was to increase our understanding of the specific contribution of  $O_2^-$  to the hyper-responsive neutrophil phenotype associated with periodontal bacteria (*P. gingivalis* & *F. nucleatum*) and any changes related to successful periodontal therapy. Phorbol 12-myristate 13-acetate (PMA) and PBS (vehicle control) were also included as positive and negative controls respectively. Previous studies have shown increased Fc $\gamma$ R and *E. coli* LPS-stimulated lucigenin-detectable  $O_2^-$  release in chronic periodontitis but none have reported responses to un-opsonised putative periodontal pathogens (Zekonis *et al.* 2003, Zilinskas *et al.* 2011).

## 5.2 UNSTIMULATED NEUTROPHIL SUPEROXIDE RELEASE

Baseline  $O_2^-$  release from patient and control neutrophils is shown in Figure 5.1(A, C). Pre-therapy  $O_2^-$  release from patient neutrophils (median 2,152RLU; range 181-7,746RLU) was significantly greater than controls (median 846RLU; range 309-3,634RLU;  $P=0.011$ ). However, at the 2-month post-therapy review, there was no significant difference in  $O_2^-$  release between patient (median 2,505RLU; range 692-6,300RLU) and control neutrophils (median 2,130RLU; range 834-5,547RLU;  $P=0.3124$ ; Figures 5.1B, D).



**Figure 5.1. Unstimulated neutrophil extracellular superoxide release at baseline (A, C; n=20) and review (B, D; n=19) measured by lucigenin-enhanced chemiluminescence (relative light units; RLU).** Box and whisker plots (A, B) showing median, interquartile range, maximum and minimum values. Line charts (C, D) showing paired patient and control neutrophils. *P*-values calculated using two-tailed Wilcoxon matched pairs test.

### 5.3 STIMULATED NEUTROPHIL SUPEROXIDE RELEASE

The overall changes in neutrophil  $O_2^-$  release in the presence of *P. gingivalis*, *F. nucleatum* and PMA compared with PBS control at baseline and 2-month review are shown in Table 5.1. As expected, neutrophils released significantly greater amounts of extracellular  $O_2^-$  after stimulation with *P. gingivalis*, *F. nucleatum* and PMA compared to PBS vehicle control ( $P<0.05$ ). The amount of neutrophil  $O_2^-$  release differed significantly between stimuli ( $P=0.0001$ ). Whilst there were no significant differences between *P. gingivalis* and *F. nucleatum* stimulated  $O_2^-$  release, PMA released approximately 3-6 times more  $O_2^-$  compared to bacterial stimuli ( $P<0.001$ ; Tables 5.2).


Stimulus	Change in lucigenin-enhanced chemiluminescence compared with PBS control			
	Baseline		Review	
	Patient	Control	Patient	Control
	Friedman $P=0.0001$	Friedman $P=0.0001$	Friedman $P=0.0001$	Friedman $P=0.0001$
<i>P. gingivalis</i>	↑*	↑*	↑***	↑*
<i>F. nucleatum</i>	↑***	↑***	↑***	↑***
PMA	↑***	↑***	↑***	↑***

**Table 5.1. Confirmation of stimulation of superoxide release detected by lucigenin-enhanced chemiluminescence by patient and control neutrophils (n=20 baseline; n=19 review) in the presence of *P. gingivalis* (MOI 100:1), *F. nucleatum* (MOI 100:1), and PMA (5nM). ↑ increase or ↔ no change in chemiluminescence compared to PBS control. *P*-values calculated using Friedman test followed by Dunn's multiple comparisons test for comparisons with PBS control: \*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$ . The RLU data upon which these changes are based are shown in Tables 5.2 & 5.3.**

BASELINE	Patient				Control			
	PBS	Pg	Fn	PMA	PBS	Pg	Fn	PMA
Median	2,669	4,028	5,603	15,026	1,077	2,024	4,049	14,224
Max	7,333	11,009	10,572	24,328	4,633	5,230	7,142	21,241
Min	234	500	1,707	6,850	286	494	1,599	2,189
Friedman test	Within patient & control groups							
$P=0.0001$								
Dunn's test	Within patient & control groups							
ns								
$P<0.001$								
$P<0.001$								
Wilcoxon test	Between patient and control groups							
ns								
$P=0.0266$								
$P=0.0064$								
ns								

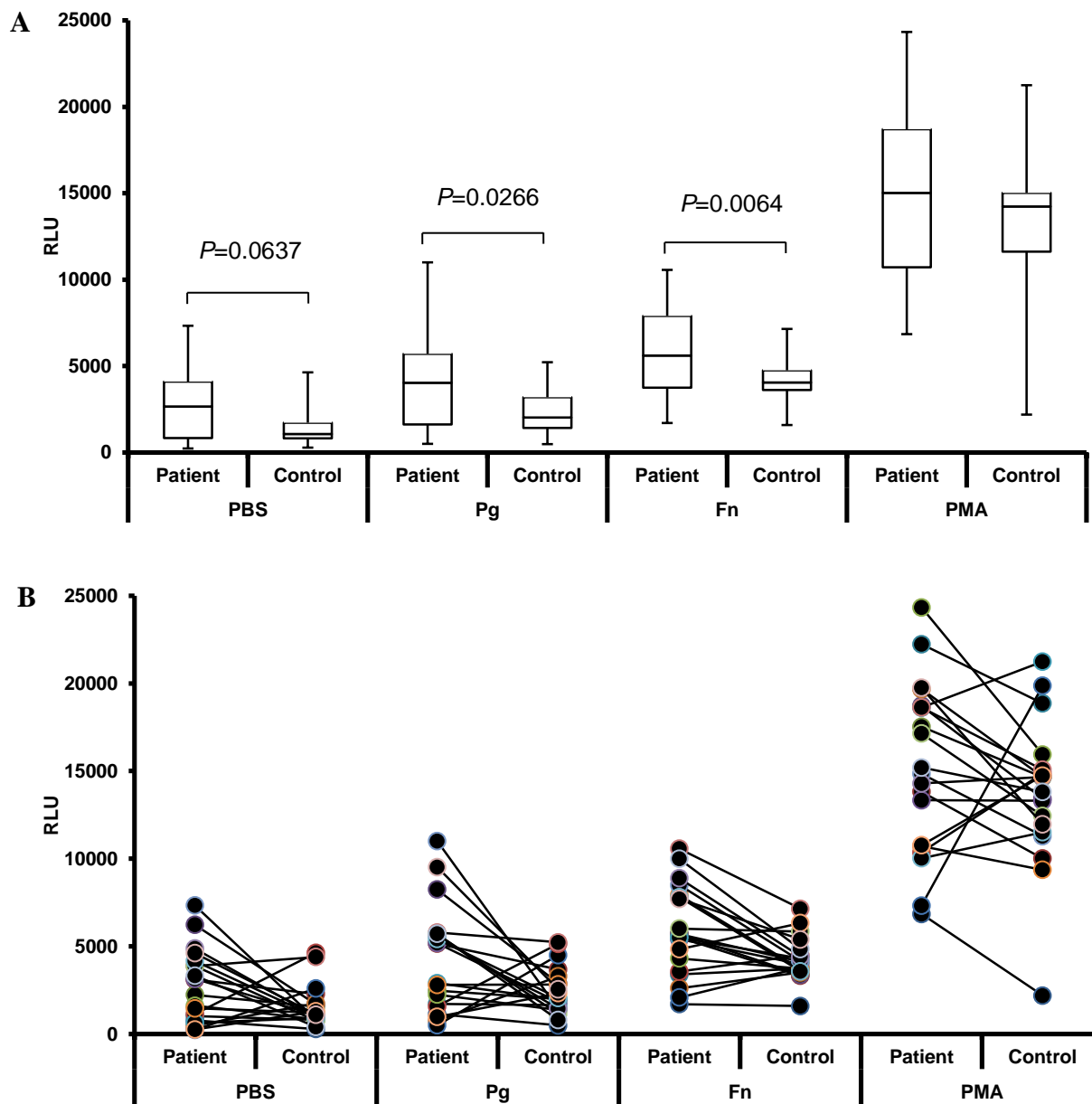
2-MONTH REVIEW	Patient				Control			
	PBS	Pg	Fn	PMA	PBS	Pg	Fn	PMA
Median	2,103	4,492	6,138	14,712	2,468	5,054	6,242	14,321
Max	6,228	11,336	8,348	18,937	4,965	9,840	8,462	22,235
Min	568	1,362	2,371	9,826	888	1,629	4,527	9,413
Friedman test	Within patient & control groups							
$P<0.0001$								
Dunn's test	Within patient & control groups							
ns								
$P<0.001$								
$P<0.001/P<0.01$								
Wilcoxon test	Between patient and control groups							
ns								
ns								
ns								
ns								

**Table 5.2. Baseline and review superoxide release from patient and control neutrophils (n=19) in the absence (PBS control) and following stimulation with *P. gingivalis* (MOI 100:1), *F. nucleatum* (MOI 100:1) and PMA (5nM). Data presented as median relative light units, maximum and minimum values. Differences between stimuli, within test groups, performed by Friedman test followed by Dunn's multiple comparisons test. Differences between patient and controls for each stimulus calculated using 2-tailed Wilcoxon matched pairs test (groups compared in table = , ns = not significant).**

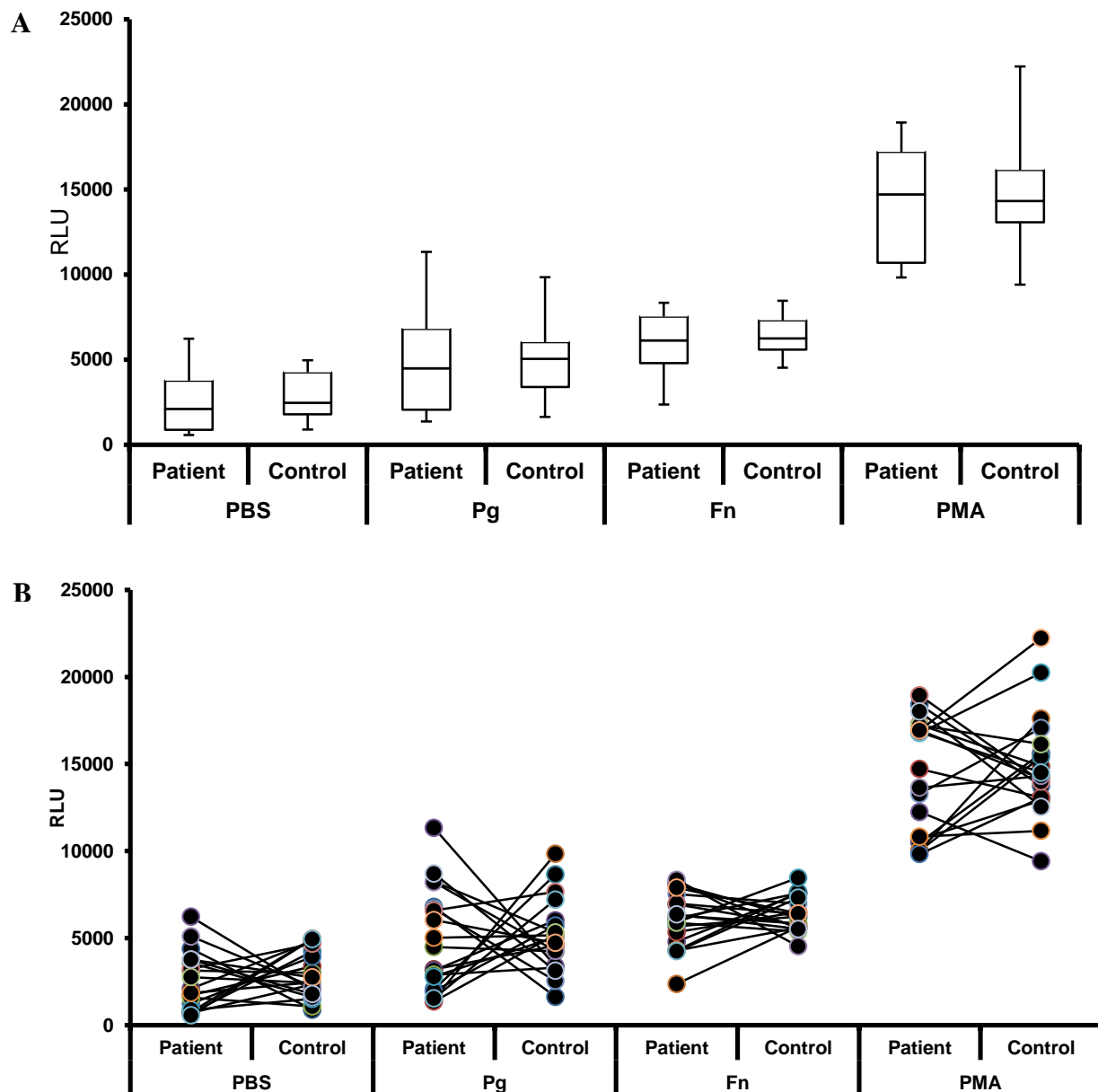


Baseline stimulated  $O_2^-$  release from patient and control neutrophils is shown graphically in Figure 5.2. Overall, patient neutrophils released significantly more  $O_2^-$  compared to controls when comparing the combined data for all stimuli ( $P=0.0002$ ). Analysis of the data for individual stimuli demonstrated that patient neutrophils released significantly more  $O_2^-$  compared with healthy controls following stimulation with *P. gingivalis* ( $P=0.0266$ ) and *F. nucleatum* ( $P=0.0064$ ) but not PMA. In response to addition of PBS (vehicle control), median levels of  $O_2^-$  release were higher from patient neutrophils compared with those from control cells but the difference did not reach statistical significance ( $P=0.0637$ ). However, these data are consistent with the greater  $O_2^-$  release by patient cells at baseline when measured without addition of anything other than the chemiluminescence assay reagents (i.e. unstimulated cells;  $P=0.011$ ; Figure 5.1). This failure to detect a statistically significant difference in  $O_2^-$  production between patient and control cells is likely due to the addition of PBS resulting in a small increase in neutrophil  $O_2^-$  release compared to the baseline unstimulated data for both patient (median increase from 2,152 to 2,669RLU) and control neutrophils (median increase from 846 to 1,077RLU) thereby reducing the differences in unstimulated  $O_2^-$  release following addition of PBS.

At the 2-month post-treatment review, there were no significant differences in stimulated extracellular  $O_2^-$  release between patients and control neutrophils when comparing data for all stimuli ( $P=0.8295$ ). In addition, there were no significant differences in neutrophil extracellular  $O_2^-$  release between patients and controls when analysing data for the individual stimuli (Table 5.2 & Figure 5.3).



**Figure 5.2. Baseline stimulated neutrophil extracellular superoxide release by patient and control neutrophils measured by lucigenin enhanced chemiluminescence (relative light units; RLU) (n=20) in the absence (PBS control), or presence of *P. gingivalis* (MOI 100:1), *F. nucleatum* (MOI 100:1) and PMA (5nM). Box and whisker plot (A) showing median, interquartile range, maximum and minimum values. Line chart (B) showing paired patient and control neutrophils. *P*-values calculated using two-tailed Wilcoxon matched pairs test.**



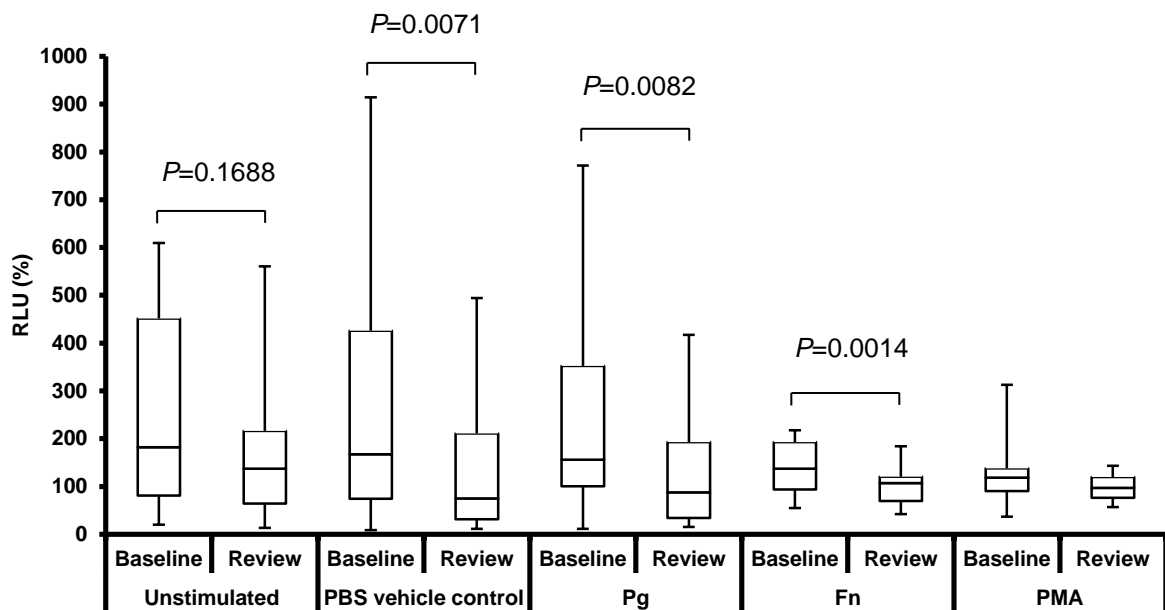
**Figure 5.3. Review stimulated neutrophil extracellular superoxide release by patient and control neutrophils measured by lucigenin enhanced chemiluminescence (relative light units; RLU) (n=19) in the absence (PBS control), or presence of *P. gingivalis* (MOI 100:1), *F. nucleatum* (MOI 100:1) and PMA (5nM). Box and whisker plot (A) showing median, interquartile range, maximum and minimum values. Line chart (B) showing paired patient and control neutrophils. There were no significant differences between patient and control volunteers using two-tailed Wilcoxon matched pairs test.**

#### **5.4 EFFECT OF PERIODONTAL THERAPY ON NEUTROPHIL SUPEROXIDE RELEASE**

An overall summary of the differences in neutrophil superoxide release between patients and controls at baseline and review is shown in Table 5.3. Paired data analysis of the lucigenin chemiluminescence results show that, 2 months post therapy, the greater release of  $O_2^-$  by patient cells compared to those from controls seen at baseline is no longer present. The change in patient  $O_2^-$  release, as a percentage of control, demonstrates a significant reduction at review compared to baseline for PBS treated cells (vehicle control), *P. gingivalis* and *F. nucleatum* stimulated cells (Figure 5.4). Interestingly, the  $O_2^-$  response of patients' cells to PMA, a receptor-independent protein kinase C activator, was similar to that of control cells at baseline and review.

Condition		Differences in neutrophil superoxide release between patient and control volunteers	
		Baseline	Review
Unstimulated		↑ ( $P=0.011$ )	↔
Stimulated	PBS	↔ ( $P=0.0637$ )	↔
	<i>P. gingivalis</i>	↑ ( $P=0.0266$ )	↔
	<i>F. nucleatum</i>	↑ ( $P=0.0064$ )	↔
	PMA	↔	↔

**Table 5.3. Summary of the differences in neutrophil superoxide release between patients and controls at baseline and review without stimulation and in the presence of additional PBS (“unstimulated” vehicle control), *P. gingivalis* (MOI 100:1), *F. nucleatum* (MOI 100:1) and PMA (5nM). ↑ signifies patient>control and ↔ signifies no difference. *P*-values calculated using two-tailed Wilcoxon matched pairs test**



**Figure 5.4. Effect of periodontal treatment on unstimulated and stimulated superoxide release by patient neutrophils (n=19) measured by lucigenin-enhanced chemiluminescence (relative light units; RLU) without stimulation and in the presence of additional PBS (“unstimulated” vehicle control), *P. gingivalis* (MOI 100:1), *F. nucleatum* (MOI 100:1) and PMA (5nM). Data presented as patient RLU as a percentage of paired control RLU. Box and whisker plot showing median, interquartile range, maximum and minimum values. *P*-value calculated using two-tailed Wilcoxon matched pairs test.**

## 5.5 DISCUSSION

The data presented in this chapter demonstrates that peripheral blood neutrophils isolated from untreated patients with chronic periodontitis release greater amounts of  $O_2^-$  compared to healthy controls in the presence and absence of periodontal pathogens. While greater production of  $O_2^-$  by unstimulated neutrophils has been reported (Guarnieri *et al.* 1991), this is the first study to demonstrate neutrophil hyper-reactivity with respect to  $O_2^-$  release following stimulation with the periodontal pathogens *P. gingivalis* and *F. nucleatum*. The current work also demonstrates that successful non-surgical therapy removed this  $O_2^-$  unstimulated hyper-activity and periodontal pathogen-stimulated hyper-reactivity. Together, the data are consistent with current literature demonstrating neutrophil hyperactive and –reactive ROS release in the pathogenesis of chronic periodontitis, along with providing supporting data that this is corrected following successful periodontal therapy.

Whilst ROS generation is essential for neutrophil host defence, both hyper-active and hyper-reactive neutrophil ROS release is a well-established pathological feature and is considered responsible for causing collateral host tissue damage in the pathogenesis of chronic periodontitis (reviewed by Chapple & Matthews 2007). This especially relates to extracellular rather than intracellular ROS release, due to the increased risk of collateral host tissue damage with extracellular ROS release. The data presented in this thesis is consistent with current literature showing hyper-active neutrophil extracellular ROS generation in the absence of overt stimulation in chronic periodontitis (Guarnieri *et al.* 1991, Matthews *et al.* 2007). Two other studies have reported greater  $O_2^-$  release from patient compared to control cells using lucigenin-enhanced chemiluminescence and buffy coat preparations rather than isolated neutrophils (Zekonis *et al.* 2003, Zilinskas *et al.* 2011). In contrast to the extracellular ROS hyperactivity of patient neutrophils, luminol-based studies have failed to detect a significant upregulation of unstimulated intracellular ROS generation in chronic periodontitis (Matthews *et al.* 2007).

Greater neutrophil  $O_2^-$  release was observed, for the first time, following stimulation with the periodontal pathogens *P. gingivalis* and *F. nucleatum*. This represents novel data specific to  $O_2^-$  release, and is consistent with neutrophil intra- and extracellular ROS hyper-reactivity following *F. nucleatum* and FcγR stimulation in chronic periodontitis (Gustafsson & Asman

1996, 1997, Fredriksson *et al.* 1998, 1999, 2003, Matthews *et al.* 2007). This data is similarly in agreement with previous studies specifically demonstrating greater  $O_2^-$  release from patient compared to control neutrophils following  $Fc\gamma R$  and *E. coli* LPS stimulation (Zekonis *et al.* 2003, Zilinskas *et al.* 2011). The lack of difference in  $O_2^-$  release between patient (untreated or treated) and control neutrophils following stimulation with PMA presented in this thesis is also consistent with the only other study that has investigated this receptor-independent activator of protein kinase C (PKC; Guarnieri *et al.* 1991). Whilst PMA stimulation resulted in a 3-6 fold greater superoxide release compared to unopsonised bacterial stimuli, the lack of hyper-reactivity when comparing patient and control responses following PMA stimulation contrasts with the hyper-reactivity in the presence of bacterial stimulation with *P. gingivalis* and *F. nucleatum*. This suggests that dysregulated receptor and downstream signalling relating to NADPH and the formation of neutrophil ROS plays a role in the pathogenesis of chronic periodontitis rather than solely the NADPH oxidase itself, since PMA bypasses receptor signalling with direct stimulation of NADPH via PKC. This is further supported by previous data demonstrating no significant difference in neutrophil NADPH phox gene expression between patients with chronic periodontitis and healthy controls (Matthews *et al.* 2007), whilst NADPH oxidase polymorphisms are considered a risk factor for aggressive periodontitis (Nibali *et al.* 2006).

The data presented here demonstrate that  $O_2^-$  hyperactivity and  $O_2^-$  hyper-reactivity to periodontal pathogens exhibited by patient neutrophils was not present after successful periodontal therapy. Whilst it is impossible to compare chemiluminescence data gathered at different time points, due to the inherent day-to-day variability in the method, it is not unreasonable to suggest that therapy has reduced the  $O_2^-$  responses of patient neutrophils such that they are no longer higher than those of cells isolated from matched controls. There have been many studies showing that neutrophils from untreated periodontitis patients consistently show significantly higher ROS generation and release than cells isolated from periodontally and medically healthy controls (Gustafsson & Asman 1996, Fredriksson *et al.* 1998, 1999, 2003, Zekonis & Zekonis 2004, Matthews *et al.* 2007). The review data in this study therefore strongly suggests that the  $O_2^-$  hyperactivity and hyper-reactivity of peripheral neutrophils in periodontitis patients is corrected by successful periodontal therapy. To date there has been only one other published longitudinal study investigating the effect of

periodontal treatment on neutrophil ROS responses which used luminol and isoluminol chemiluminescence methods to detect intra- and extracellular ROS respectively, rather than extracellular  $O_2^-$  (Matthews *et al.* 2007). This latter study demonstrated persistence of unstimulated, iso-luminol detectable, extracellular ROS hyperactivity but correction of luminol-detectable, FcγR-stimulated intracellular ROS hyper-reactivity following treatment. Thus, the data presented in this chapter demonstrating that TLR-stimulated neutrophil  $O_2^-$  release was similar to that of controls at review following periodontal treatment are similar to the corrected FcγR-stimulated total ROS generation previously reported post therapy (Matthews *et al.* 2007). However the unstimulated extracellular  $O_2^-$  release data presented in this chapter differs from the previously reported persistent unstimulated extracellular ROS release detected by isoluminol, which detects predominantly extracellular,  $H_2O_2$ -derived, HOCl (Matthews *et al.* 2007). One possible explanation may relate to increased intracellular levels of  $H_2O_2$  due to upregulated enzymatic conversion of  $O_2^-$  by intracellular SOD and/or a reduction in its intracellular transformation to HOCl and water by MPO and catalase respectively (Figure 5.5). As  $H_2O_2$  can be transported across cell membranes, increased intracellular levels could then result in increased extracellular levels detectable as HOCl in the isoluminol assay that was used to show persistent hyperactivity following periodontal treatment (Matthews *et al.* 2007). Whilst MPO and SOD polymorphisms have been identified in pancreatic adenocarcinoma (Wheatley-Price *et al.* 2008) and chronic pelvic pain syndrome (Arisan *et al.* 2006), there are currently no known published studies investigating MPO, SOD or catalase activity in patients with chronic periodontitis.

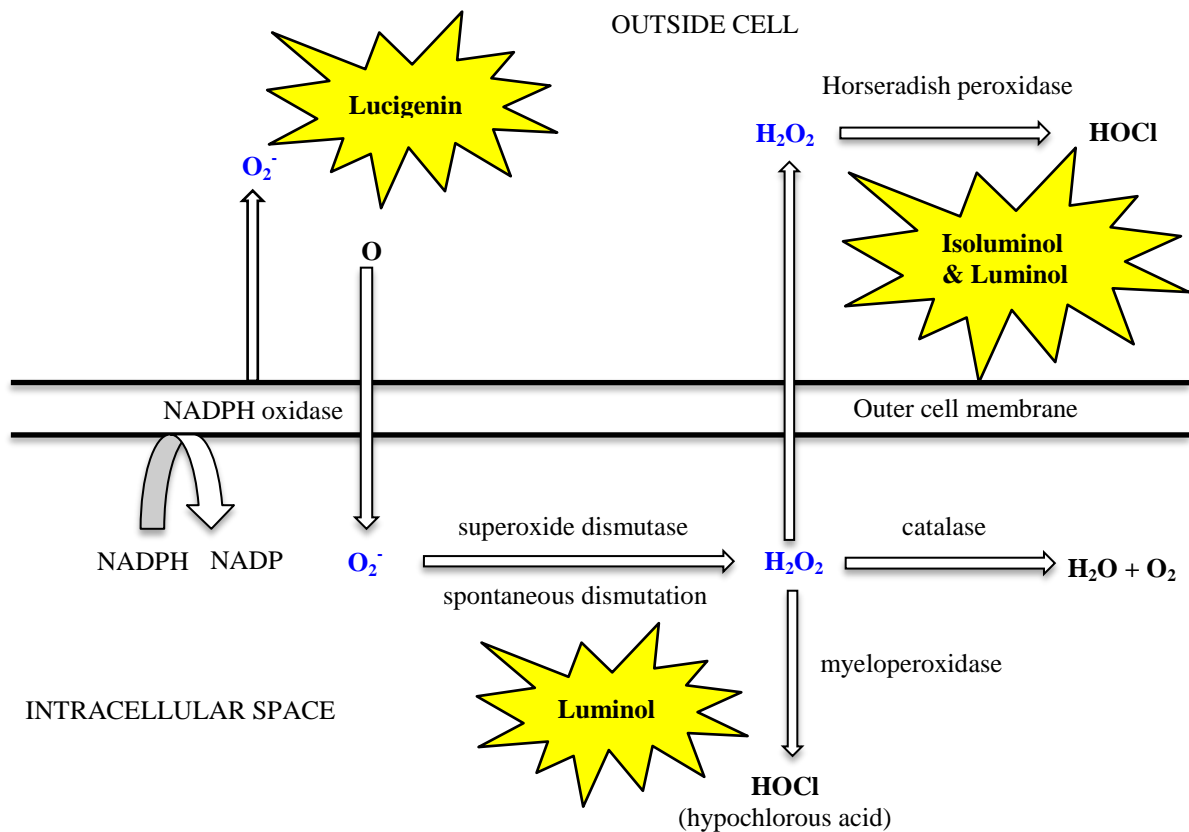
Exaggerated neutrophil extracellular  $O_2^-$  release will have a number of different effects on the host response in the pathogenesis of chronic periodontitis. Greater  $O_2^-$  release into the surrounding tissue will have the potential to directly cause endothelial cell damage (Droy-Lefaix *et al.* 1991), lipid peroxidation (Dix & Aikens 1993) and DNA strand breaks (Dix *et al.* 1996). Furthermore, whilst the  $O_2^-$  radical has relatively little antimicrobial activity and toxicity in itself, this is increased by its conversion to downstream  $H_2O_2$  and HOCl in the presence of SOD and MPO respectively (Babior 1999, Dahlgren *et al.* 1999). Taken together, increased neutrophil ROS release will have the potential to increase NET (Palmer *et al.* 2012) and cytokine release (Ndengele *et al.* 2005), as well as affecting chemotactic potential (Deitch *et al.* 1990). The median  $O_2^-$  release for unstimulated and *F. nucleatum* and *P. gingivalis*



stimulated cells, expressed as a percentage of patient compared to matched healthy control neutrophils, was 182, 137 and 156% respectively. However, this is likely to be under-representative of the *in-vivo* impact regarding the pathogenesis of chronic periodontitis given the relatively large numbers of neutrophils recruited as part of the inflammatory-immune response. Furthermore, the greater cytokine release from patient neutrophils presented in this thesis (Chapter 4) may further exacerbate neutrophil ROS release (Dias *et al.* 2011, Fredriksson *et al.* 2012, Figure 4.6). The presence of oxidative stress represents an important risk factor regarding chronic periodontitis and co-morbidity including type-2 diabetes (Karima *et al.* 2005, Chapple & Genco 2013), atherogenic vascular disease (Dietrich *et al.* 2013), rheumatoid arthritis (dePablo *et al.* 2009) and ischaemic stroke (Scannapieco *et al.* 2003). This is further emphasised by the correction of neutrophil extracellular  $O_2^-$  hyperactivity and –reactivity following periodontal treatment presented in this thesis, in that treatment then reduces peripheral blood neutrophil  $O_2^-$  release to that of healthy controls, therefore reducing the risk of co-morbidity following periodontal treatment. Furthermore, this data adds to our current evidence that chronic periodontitis is a modifiable risk factor for the development of other systemic inflammatory diseases where oxidative stress is a pathological feature.

The relative contribution of greater neutrophil ROS release to oxidative stress in the pathogenesis of chronic periodontitis and systemic disease is further compounded by the presence of antioxidants and the redox balance of both the neutrophil and the surrounding tissues (Brock *et al.* 2004, Chapple *et al.* 2013). The redox state of the neutrophil (GSH:GSSG ratio) has been shown to be altered in chronic periodontitis leading to cellular stress resulting in the relocation of NADPH oxidase to the outer cell membrane (Dias *et al.* 2013) which may partly explain the increased extracellular  $O_2^-$  release in chronic periodontitis presented in this thesis. Therefore, the increased release of extracellular  $O_2^-$  may be an inherently protective cellular event ensuring neutrophil survival in the presence of oxidative stress, with the likely consequence of collateral host tissue damage. The association between low antioxidant defence and increased biomarkers of oxidative damage in periodontitis plasma has been investigated in several studies (reviewed by Chapple & Matthews 2007). Furthermore, the redox disturbances in periodontitis neutrophils are associated with dysregulation of the anti-inflammatory transcription factor Nrf2 (Dias *et al.* 2013). In

addition, sulforaphane, which is a natural product found in cruciferous vegetables, has been shown to restore cellular glutathione levels and thereby reduce the hyperactivity of neutrophils associated with chronic periodontitis highlighting the potential importance of a healthy diet and antioxidant intake in the management of chronic periodontitis and also in reducing the risk of systemic disease associated with oxidative stress (Chapple *et al.* 2009, Dias *et al.* 2013).



**Figure 5.5. Overview of the neutrophil respiratory burst including generation of  $O_2^-$  and subsequent conversion to downstream ROS.** The corrected unstimulated extracellular  $O_2^-$  release hyperactivity presented in this thesis differs from the previously reported persistent  $H_2O_2$ -derived HOCl hyperactivity following periodontal treatment (Matthews *et al.* 2007). One possible explanation may relate to increased levels and extracellular release of  $H_2O_2$  due to upregulated enzymatic conversion of  $O_2^-$  by intracellular SOD and/or reduced transformation by MPO/catalase.

Overall, the neutrophil  $O_2^-$  release data presented in this thesis is consistent with current literature with regards to hyperactive and –reactive neutrophil ROS generation in the pathogenesis of chronic periodontitis. Taken together with a body of literature demonstrating oxidative changes in peripheral blood of patients with systemic diseases such as diabetes (Allen *et al.* 2011), and together with those previously published (Matthews *et al.* 2007), these data suggest that chronic periodontitis is a modifiable risk factor, the successful treatment of which has the potential to reduce the risk of co-morbidity associated with oxidative stress due neutrophil ROS generation and release.

# **CHAPTER 6**

## **RESULTS & DISCUSSION**

**Case-control study:**

**Baseline differences in neutrophil extracellular trap (NET) release  
& chemotactic accuracy in chronic periodontitis**

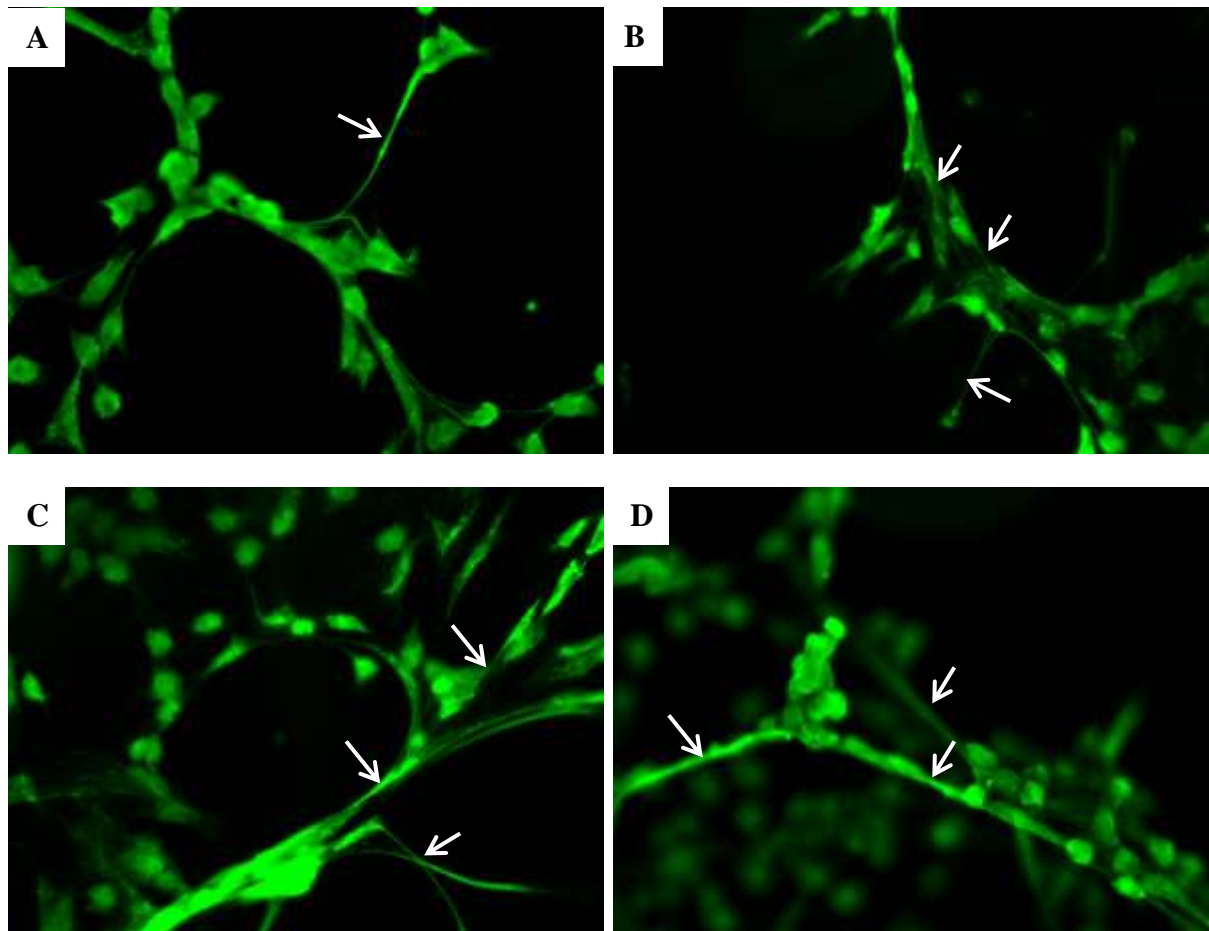
## **6.1 INTRODUCTION**

Isolated neutrophils from untreated patient and healthy control volunteers were cultured in the presence and absence of known stimuli for neutrophil extracellular trap (NET; section 2.2.9) release (PMA and HOCl) to determine, for the first time, whether dysregulated NET release is involved in the pathogenesis of chronic periodontitis. In addition, directional chemotactic accuracy of patient and control neutrophils in response to IL-8 and fMLP was determined using a novel direct visualisation chemotaxis chamber (section 2.2.10). Although traditional “chemotaxis” has been investigated in chronic periodontitis previously, this is the first time that this new, directional chemotaxis assay has been applied to the disease.

## **6.2 NEUTROPHIL EXTRACELLULAR TRAP RELEASE**

Neutrophil extracellular trap (NET) release was confirmed by fluorescence microscopy and quantified using a recently developed fluorometric assay (sections 2.2.9.1 & 2).

Extracellular trap release from patient and control neutrophils following stimulation with PMA or HOCl is shown in Figure 6.1. Neutrophil extracellular traps were observed to confirm their release, with no noticeable visible differences in appearance or quantity between patient and control neutrophils. Neutrophil extracellular traps were observed as fluorescent streaks alongside non-viable “NETosed” and necrosed neutrophils and cell debris thereby making it difficult to quantify NET release using direct observation of Sytox Green stained preparations. A validated fluorometric assay (section 2.2.9.2) was therefore used to quantify and determine NET release from patient and control neutrophils.




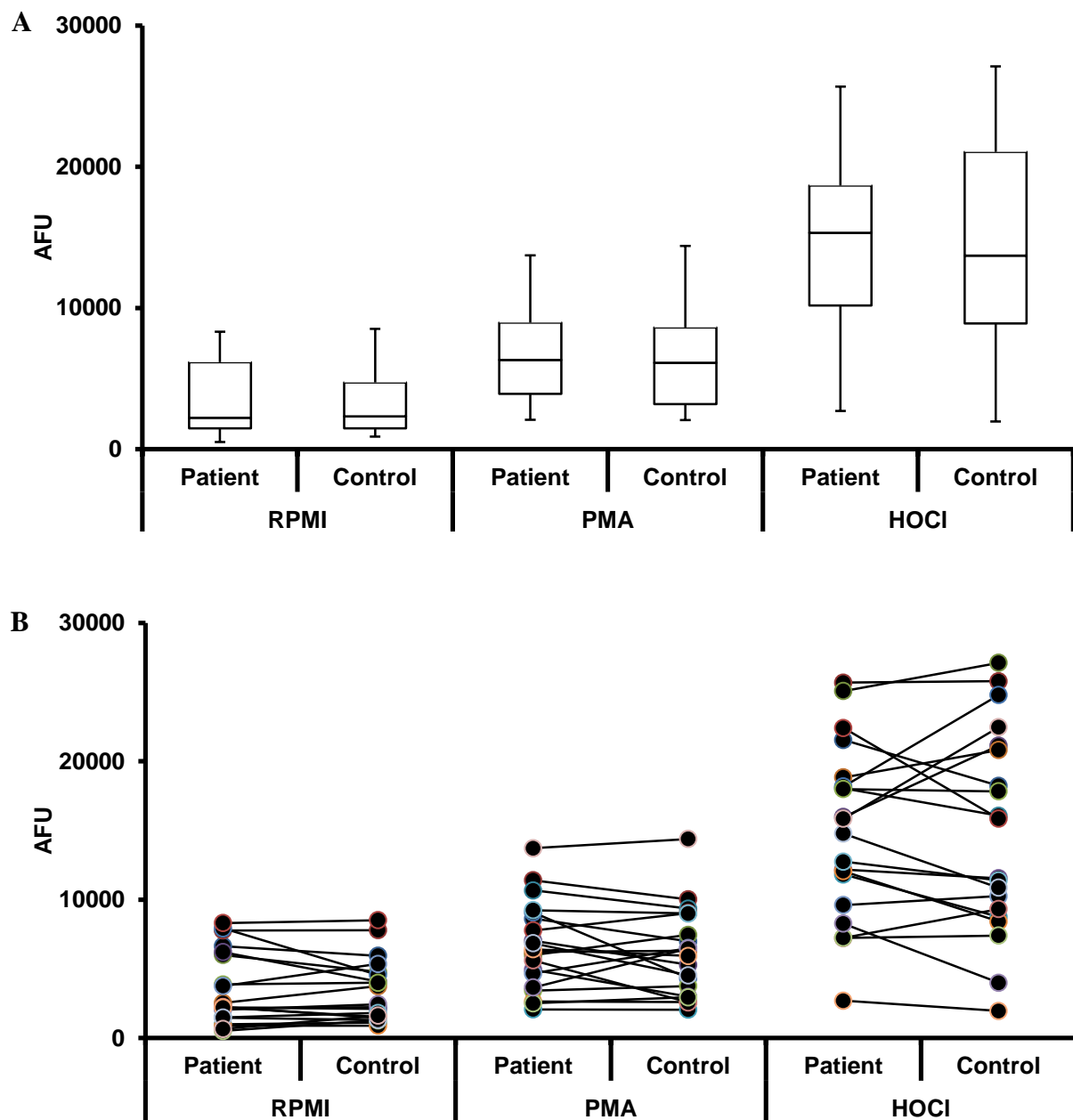
**Figure 6.1. Fluorescence microscopy of extracellular traps from patient (A, B) and control (C, D) neutrophils stained with Sytox Green following stimulation with (A, C) 25nM PMA and (B, D) 0.75mM HOCl. Please note the presence of NET-like structures as indicated by the arrows consistent with current literature (Brinkmann *et al.* 2004, Palmer *et al.* 2012). However it is impossible to reliably distinguish between NETs, non-viable NETosed and necrotic neutrophils/cell debris, thereby making it difficult to accurately determine NET quantification using direct observation of Sytox Green stained preparations.**

Both patient and control cells showed greater NET release following stimulation with PMA ( $P<0.05$ ) and HOCl ( $P<0.001$ ) compared to RPMI control, demonstrating the effectiveness of both agents to stimulate NET production (Table 6.1). Furthermore, greater NET release was observed following stimulation with HOCl compared to PMA ( $P<0.05$ ), a finding consistent with recently published data (Palmer *et al.* 2012).

There were no statistically significant differences in NET release between patient and control cells under any of the test conditions (Figure 6.2). The median and ranges of values were similar between groups and there was no tendency for values to be greater in one group (Figure 6.2B).

NET release (AFU)	Patient			Control		
	RPMI	PMA	HOCl	RPMI	PMA	HOCl
Median	2,229	6,319	15,318	2,330	6,116	13,705
Max	8,313	13,720	25,685	8,523	14,383	27,118
Min	515	2,075	2,700	895	2,053	1,957
Friedman test						
$P<0.0001$						
Dunn's test						
$P<0.05$						
$P<0.001$						
$P<0.01$						

**Table 6.1. Neutrophil extracellular trap release from patient and control neutrophils (n=20) in the absence (RPMI control) or following stimulation with PMA (25nM) or HOCl (0.75mM).** Data presented as median, maximum and minimum arbitrary fluorescence units. Within patient and control group  $P$ -values calculated using Friedman test followed by Dunn's multiple comparisons test (groups compared in table = ).



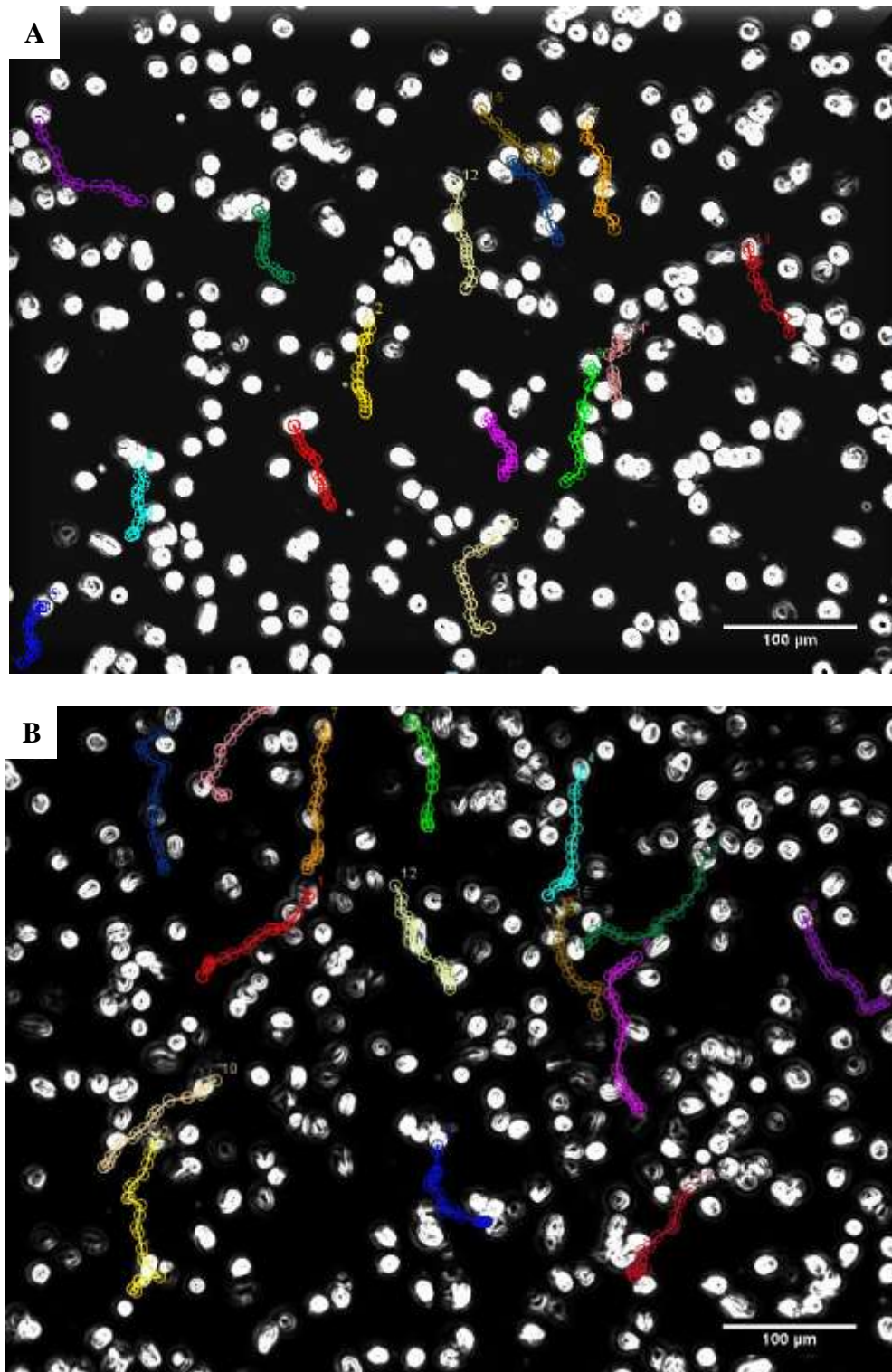
**Figure 6.2. Neutrophil extracellular trap release by patient and control neutrophils measured by fluorometric quantification (arbitrary fluorescence units; AFU; n=20) in the absence (RPMI control) or following stimulation with PMA (25nM) or HOCl (0.75mM).** (A) Box and whisker plot showing median, interquartile range, maximum and minimum values. (B) Line chart showing paired patient and control values. No significant differences were detected between patient and control cells using one-tailed Mann-Whitney test.



### **6.3 NEUTROPHIL CHEMOTACTIC ACCURACY**

Neutrophil chemotactic accuracy was determined using a novel directional chemotaxis assay (section 2.2.10) in the presence of known chemo-attractants, fMLP and IL-8. RPMI was used as a negative control. The data obtained were initially analysed qualitatively using three types of graphical plot before determining the chemotactic index, speed and velocity of cells to allow direct comparisons between patient and control cell migration.

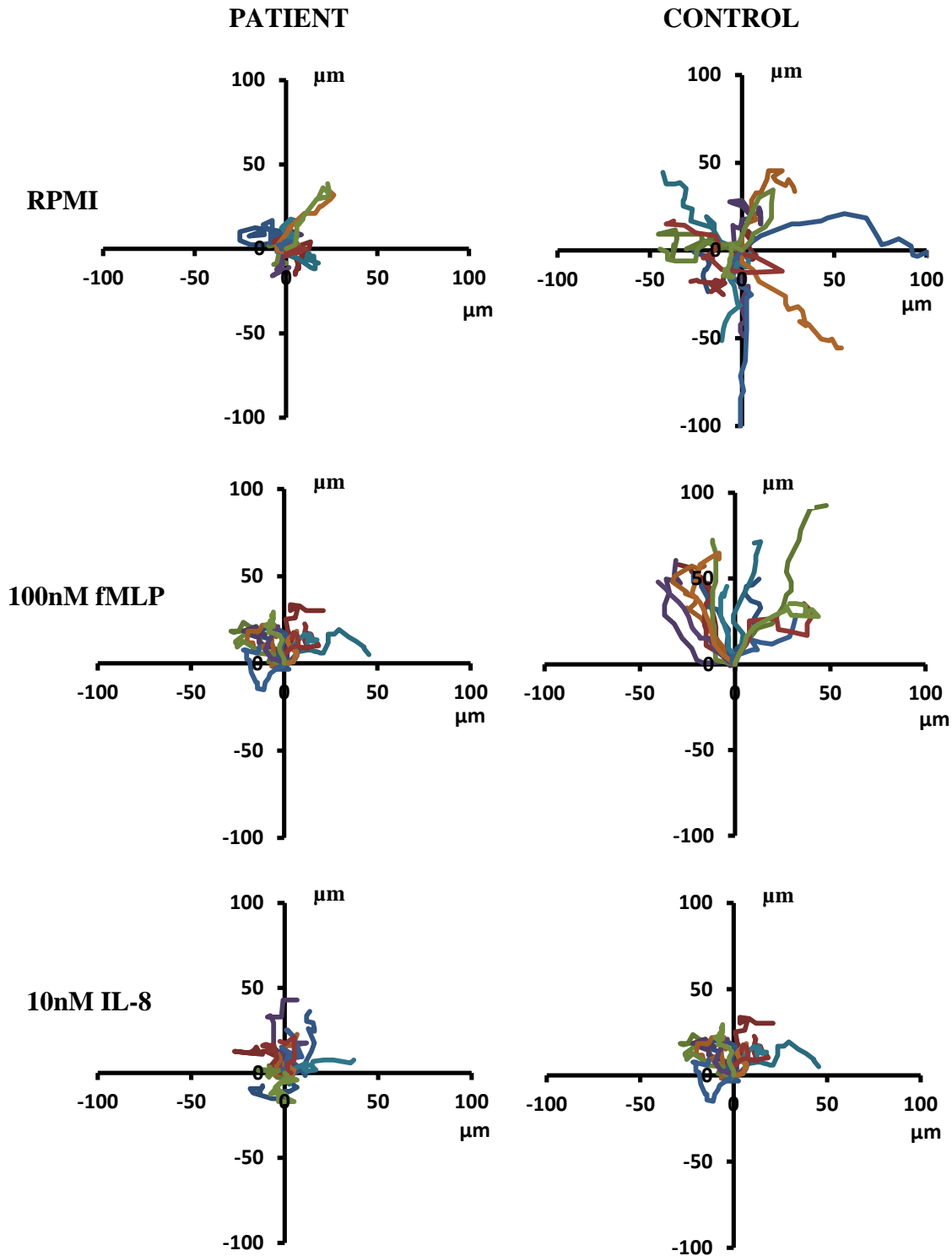
Examples of individual neutrophil migratory tracks following 20 minutes of phase time-lapse recording in the presence of fMLP are shown in Figure 6.3. Subjectively, both patient and control neutrophils demonstrated a strong bias for migration toward the source of the chemoattractant, along with a lack of directed migration in the presence of additional RPMI as a negative control. The lengths of each individual cell track represent the distance moved within the 20 minute recordings, with control neutrophils moving a generally greater distance, representative of greater speed (section 6.3.2) and velocity (section 6.3.3) of movement compared to neutrophils isolated from patients with chronic periodontitis.



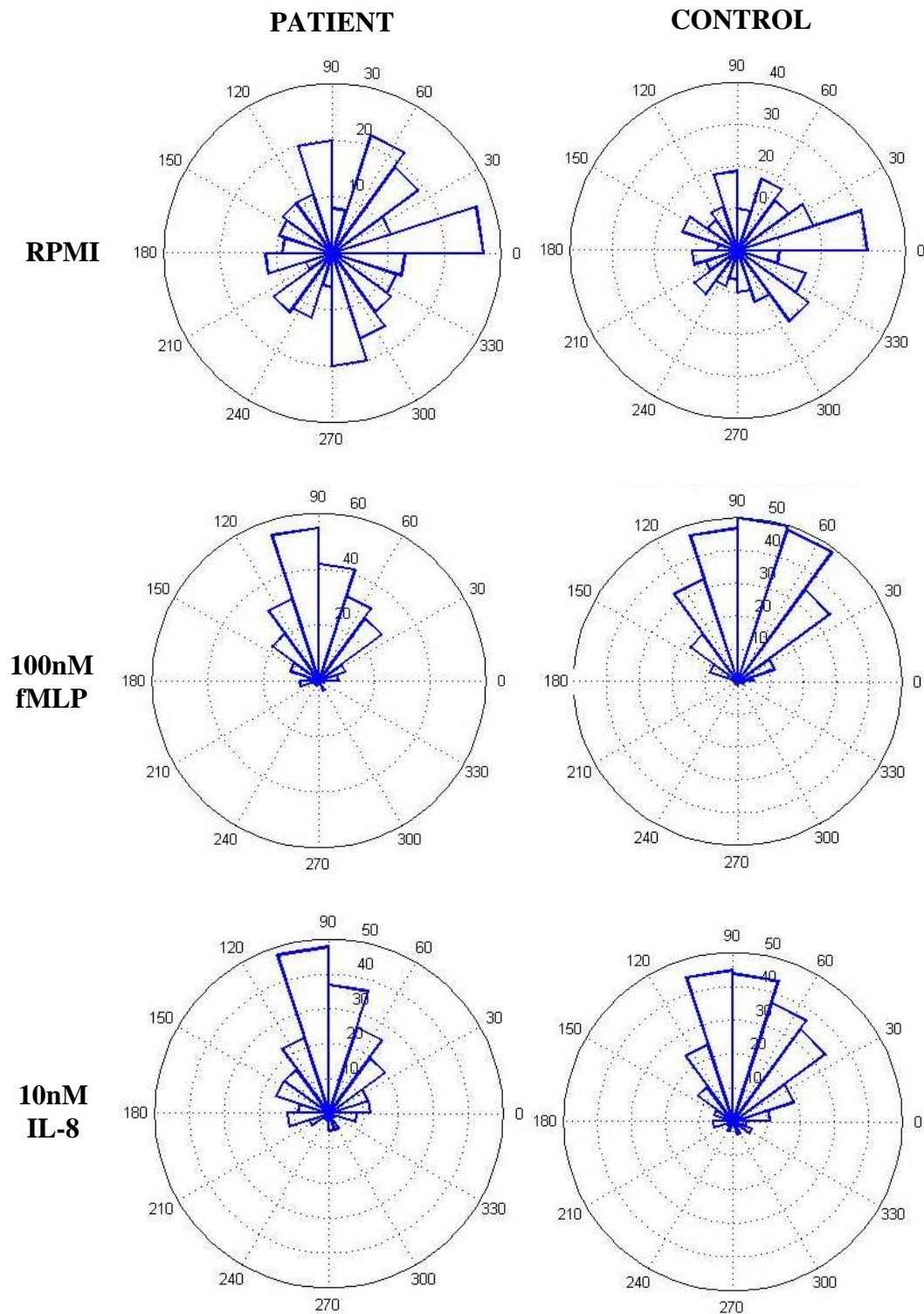
**Figure 6.3. Individual neutrophil migratory tracks towards fMLP (100nM) from a patient with chronic periodontitis (A) and healthy control (B).** The image shows the final neutrophil positions within the Insall chemotaxis chamber after 20 minutes of phase time-lapse recording. The top of the picture represents the source of the chemotactic signal. The coloured tracks indicate the path of migration for each cell analysed; each track begins where the cell was positioned at the start of recording and ends where the cell was positioned at the end of recording.

Individual neutrophil tracks in the presence of fMLP, IL-8 or RPMI control were subsequently plotted as spider diagrams calibrated to a central fixed reference point as shown in Figure 6.4. These plots confirmed direct observation of individual cell tracks (Figure 6.3), clearly showing that both patient and control neutrophil migration demonstrated a strong bias towards the chemoattractant source compared to undirected migration in the presence of additional RPMI. In addition, and similarly in agreement with the direct observation of neutrophil migration, control neutrophils generally moved a greater distance representative of greater speed and velocity compared to periodontitis neutrophils. Whilst spider diagrams are a useful way of displaying neutrophil migration per individual, the plots become less clear with the addition of greater numbers of cell tracks. Therefore, neutrophil migration for all patient and control volunteers was displayed as angular histograms and rose plots depicting clusters of neutrophil migration, demonstrating the strong bias for migration towards the source of the chemo-attractant compared to RPMI control as shown in Figure 6.5.

In order to more clearly display the direction and magnitude of the mean neutrophil migratory pattern, resultant vector plots were generated including calculation of the mean resultant vector length (section 2.2.10.2; Figure 6.6). Mean resultant vector lengths indicated that patient neutrophils, on average, were more accurately directed towards the chemoattractant source ( $90^\circ$ ) in the presence of fMLP ( $92^\circ$ ) and IL-8 ( $92^\circ$ ) compared to healthy controls ( $82^\circ$  and  $76^\circ$  for fMLP and IL-8 respectively). However, there was greater variation in neutrophil migration in the presence of fMLP and IL-8 for patient compared to control neutrophils as shown by the circles on the circumference of the rose plots and larger 95% confidence intervals (Figure 6.6). Given the greater variation in neutrophil migration, patient neutrophils had a shorter mean resultant vector length representative of impaired directional chemotactic accuracy (i.e. a smaller proportion of the cell population analysed moving in the direction of the vector line) in the presence of fMLP (0.6624) and IL-8 (0.5363) compared to healthy control neutrophils (0.8342 and 0.7027 for fMLP and IL-8 respectively; Figure 6.6).

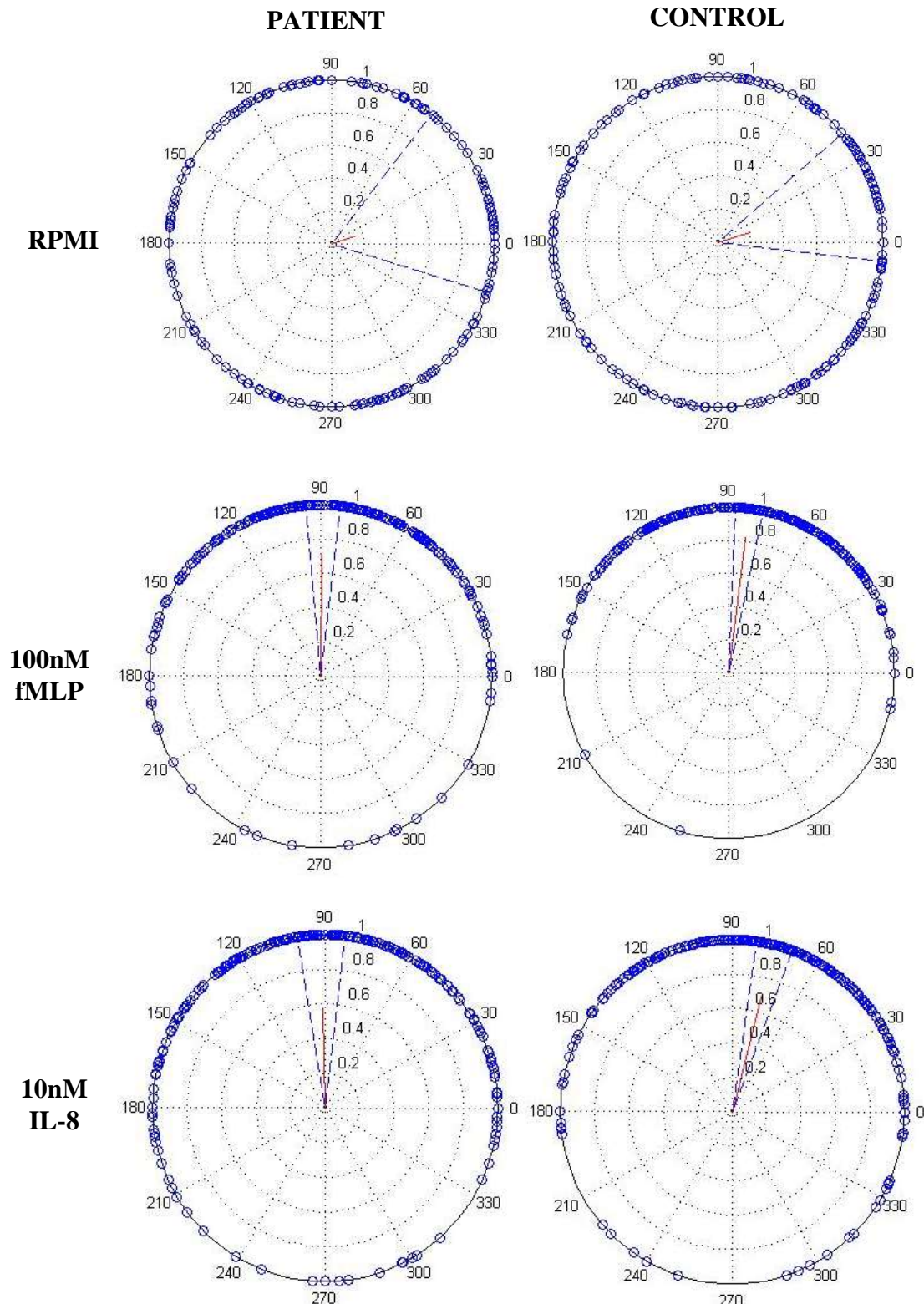


**Figure 6.4. Spider diagrams representing migration of neutrophils from a patient with chronic periodontitis and a healthy control in the presence of RPMI, fMLP (100nM) and IL-8 (10nM).** Cell tracks representative of individual neutrophil migrations ( $n=15$ ) calibrated from a central fixed reference point (0, 0) from a patient and paired control volunteer. The top of the pictures represent the source of the chemotactic signal. Note that the paths of individual neutrophils from both patient and control display a bias for migration towards the chemo-attractant.



**Figure 6.5. Angular histograms (rose plots) representing neutrophil migration for patient and control neutrophils (n=18 pairs) in the presence of RPMI, fMLP (100nM) and IL-8 (10nM).** The top of each histogram (90°) represents the source of the chemotactic signal. The width and angle of each angular histogram bar represents the proportion and migratory directions respectively of groups of neutrophils that are grouped by directionality.





**Figure 6.6. Resultant vector plots representing neutrophil migration for patient and control neutrophils (n=18 pairs) in the presence of RPMI, fMLP (100nM) and IL-8 (10nM).** The top of each histogram (90°) represents the source of the chemotactic signal. The red line represents the mean resultant vector highlighting the direction and magnitude of the mean cell migration (the longer the red line the stronger the magnitude and direction) bounded by the 95% confidence intervals. The circles on the circumference of the rose plots represent the individual end-points of neutrophil migration calibrated from the central fixed reference point.

Whilst the use of spider diagrams, angular histograms and rose plots represent novel methods to qualitatively assess cell migration, they cannot be used to objectively compare patient and control neutrophil chemotaxis. Therefore, patient and control chemotactic indices, speed and velocity of neutrophil migration were calculated to allow comparisons to be made using circular statistics (section 2.2.10.3).

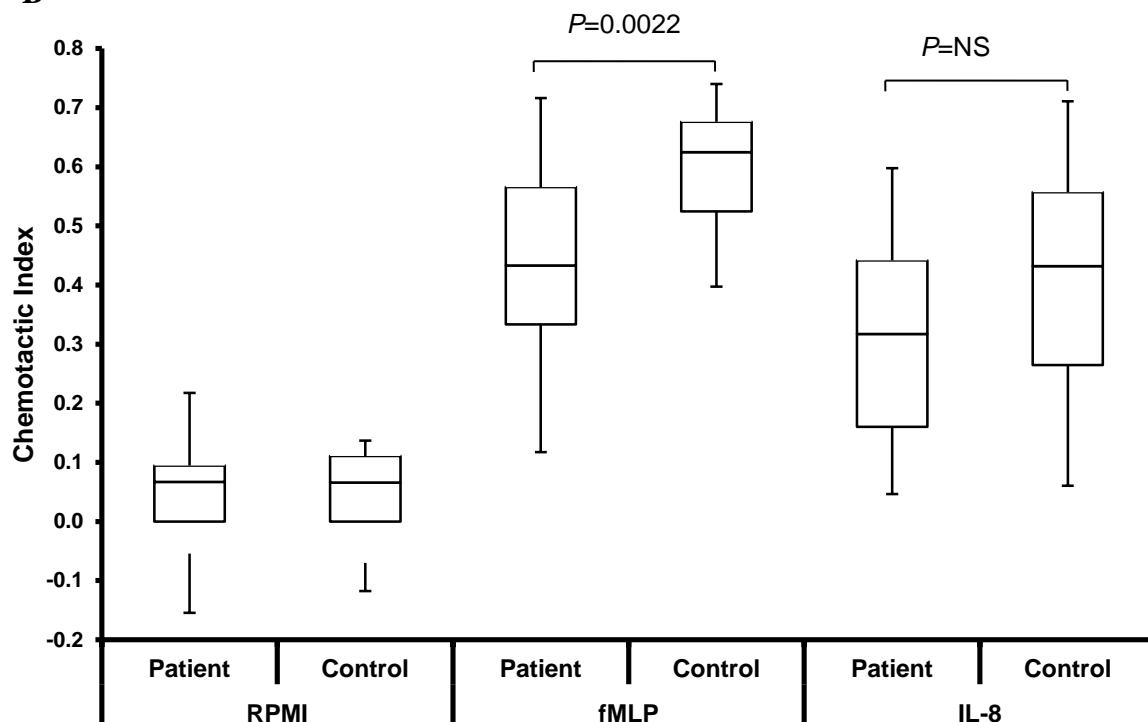
### **6.3.1 Chemotactic index**

Chemotactic index was calculated (section 2.2.10.3) as a measure of the overall accuracy of neutrophil migration towards a chemoattractant source. Patient and control neutrophil chemotactic index differed in the presence of fMLP, IL-8 and RPMI as the negative control ( $P<0.0001$ ; Figure 6.7). As expected, neutrophil chemotactic index was greater in the presence of fMLP ( $P<0.001$ ) and IL-8 ( $P<0.05$ ) compared to RPMI control for both patient and control cells. Compared to healthy controls, patient neutrophils had a reduced chemotactic index towards fMLP (median 0.43 & 0.63) and IL-8, but this difference was only statistically significant for fMLP ( $P=0.0022$ ). There was no significant difference in chemotactic index in the presence of RPMI as negative control.

**A**

Chemotactic Index	Patient			Control		
	RPMI	fMLP	IL-8	RPMI	fMLP	IL-8
Median	0.013	0.433	0.317	-0.004	0.625	0.432
Max	0.163	0.716	0.597	0.067	0.740	0.711
Min	-0.154	0.118	0.046	-0.118	0.397	0.060
<b>Friedman test</b>						
$P < 0.0001$						
<b>Dunn's test</b>						
$P < 0.001$						
$P < 0.05$						
$P < 0.05$						
ns						

**B**



**Figure 6.7. Chemotactic index of patient and control neutrophils (n=18) in the presence of RPMI, 100nM fMLP and 10nM IL-8.** Table (A) presents median, maximum and minimum values. Within patient and control group P-values calculated using Friedman test followed by Dunn's multiple comparisons test (groups compared in table =  ). Box and whisker plot (B) shows median, interquartile range, maximum and minimum values. P-values for patient versus control comparisons are calculated using two-tailed Mann-Whitney test.



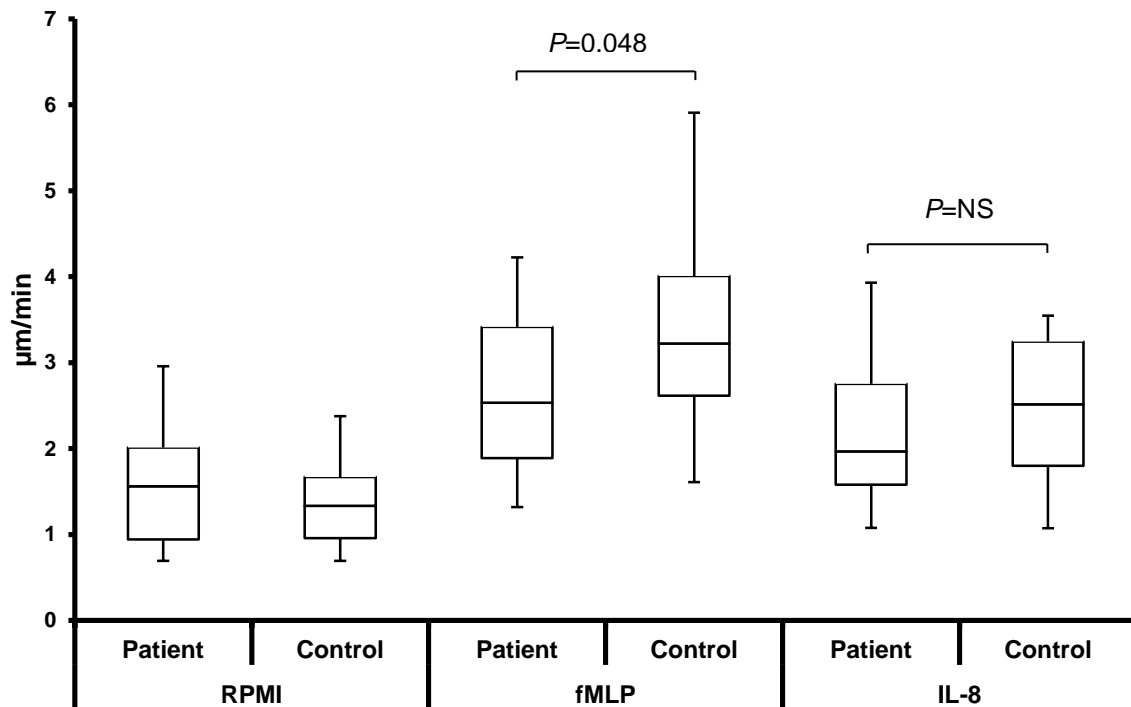
### 6.3.2 Speed

The speed of neutrophil movement differed in the presence of fMLP, IL-8 and RPMI for both patient and control volunteers ( $P \leq 0.0173$ ; Figure 6.8). Whilst control neutrophil migration speed was greater in the presence of fMLP and IL-8 compared to RPMI control ( $P < 0.05$ ), these differences were not significant for patient neutrophils. This was reflected in the finding that patient neutrophils migrated with less speed compared to controls in the presence of fMLP (median 2.53 & 3.22  $\mu\text{m}/\text{min}$ ;) and IL-8 (median 1.97 & 2.51  $\mu\text{m}/\text{min}$ ) but this reduction was only significant for fMLP ( $P = 0.048$ ). There was no significant difference in neutrophil movement speed between patient and control cells in the presence of additional RPMI as negative control.

A

Speed ( $\mu\text{m}/\text{min}$ )	Patient			Control		
	RPMI	fMLP	IL-8	RPMI	fMLP	IL-8
Median	1.560	2.533	1.968	1.333	3.223	2.514
Max	2.959	4.226	3.930	2.377	5.907	3.547
Min	0.692	1.319	1.077	0.692	1.610	1.073
<b>Friedman test</b>						
$P<0.0001$						
$P=0.0173$						
<b>Dunn's test</b>						
ns						
$P<0.01$						
ns						
$P<0.05$						
ns						

B



**Figure 6.8. Speed ( $\mu\text{m}/\text{min}$ ) of patient and control neutrophil migration (n=18) in the presence of RPMI, 100nM fMLP and 10nM IL-8.** Table (A) presents median, maximum and minimum values. Within patient and control group P-values calculated using Friedman test followed by Dunn's multiple comparisons test (groups compared in table =  ). Box and whisker plot (B) showing median, interquartile range, maximum and minimum values. P-values for patient versus control comparisons are calculated using two-tailed Mann-Whitney test.

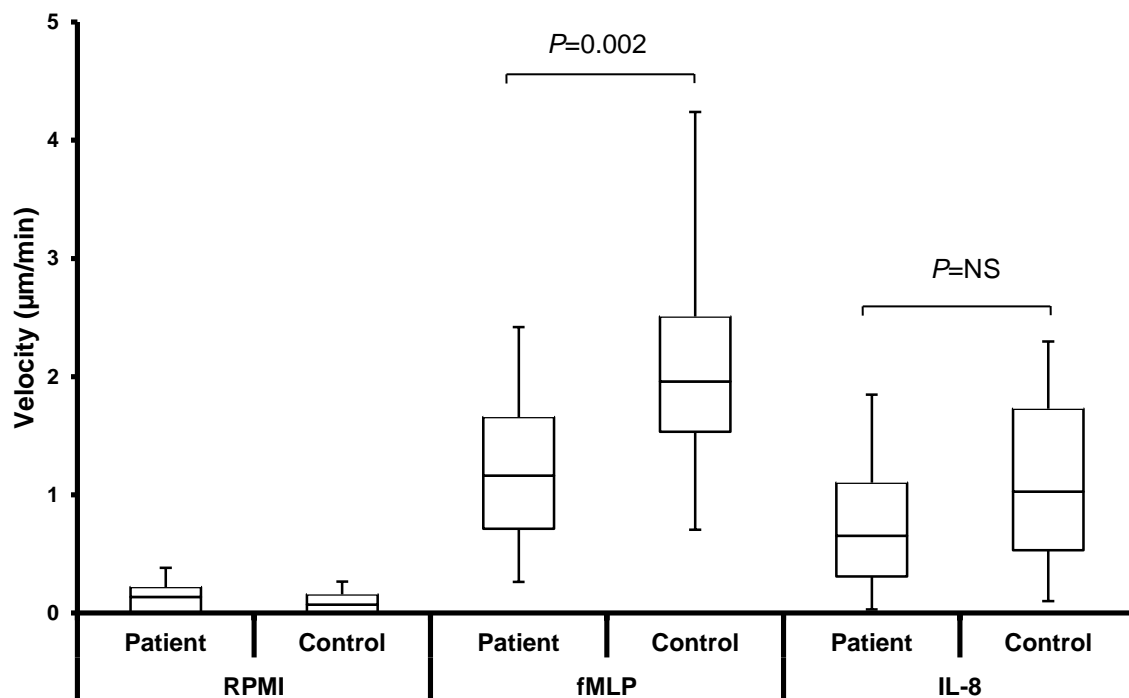
### 6.3.3 Velocity

Velocity was calculated as the speed of neutrophil migration in a consistent direction towards the source of the chemoattractant (section 2.2.10.3). Patient and control neutrophil velocity differed between fMLP, IL-8 and RPMI ( $P<0.0001$ ; Figure 6.9). Furthermore, neutrophil velocity for both patient and control cells was greater in the presence of fMLP ( $P<0.001$ ) and IL-8 ( $P<0.05$ ) compared to RPMI control. Compared to healthy controls, patient neutrophils migrated with reduced velocity in the presence of fMLP (median 1.16 & 1.96 $\mu\text{m}/\text{min}$ ) and IL-8 (median 0.65 & 1.03 $\mu\text{m}/\text{min}$ ) but, as for chemotactic index and speed, this difference was only statistically significant for fMLP ( $P=0.002$ ).

**A**

Velocity ( $\mu\text{m}/\text{min}$ )	Patient			Control		
	RPMI	fMLP	IL-8	RPMI	fMLP	IL-8
Median	0.053	1.163	0.653	0.020	1.960	1.026
Max	0.299	2.420	1.848	0.216	4.240	2.298
Min	-0.377	0.263	0.030	-0.712	0.706	0.100
<b>Friedman test</b>						
$P < 0.0001$						
<b>Dunn's test</b>						
$P < 0.001$						
$P < 0.05$						
$P < 0.05$						
ns						

**B**



**Figure 6.9.** Velocity ( $\mu\text{m}/\text{min}$ ; speed in a consistent direction towards the source of the chemoattractant) of patient and control neutrophil migration ( $n=18$ ) in the presence of RPMI, 100nM fMLP and 10nM IL-8. Table (A) presents median, maximum and minimum values. Within patient and control group  $P$ -values calculated using Friedman test followed by Dunn's multiple comparisons test (groups compared in table =  ). Box and whisker plot (B) showing median, interquartile range, maximum and minimum values.  $P$ -values for patient versus control comparisons are calculated using two-tailed Mann-Whitney test.

#### **6.3.4 Correlation between neutrophil IL-8 release & chemotactic accuracy**

Although not statistically significant, patient neutrophils displayed a lower median chemotactic index compared to healthy controls in the presence of IL-8. Data presented in this thesis has also demonstrated greater IL-8 release from patient compared to control neutrophils (section 4.5.2). Due to the possibility that exaggerated neutrophil IL-8 release by patient neutrophils may partly explain their reduced chemotactic accuracy, correlations between patient neutrophil IL-8 release and chemotactic index, speed and velocity were determined.

A low but statistically significant correlation was observed between patient neutrophil IL-8 release (unstimulated, RPMI control; section 4.5.2) and chemotactic index towards fMLP only (Figure 6.10). There were no noticeable correlations between patient neutrophil IL-8 release and either speed or velocity of neutrophil migration in the presence of fMLP and IL-8 as chemoattractants.

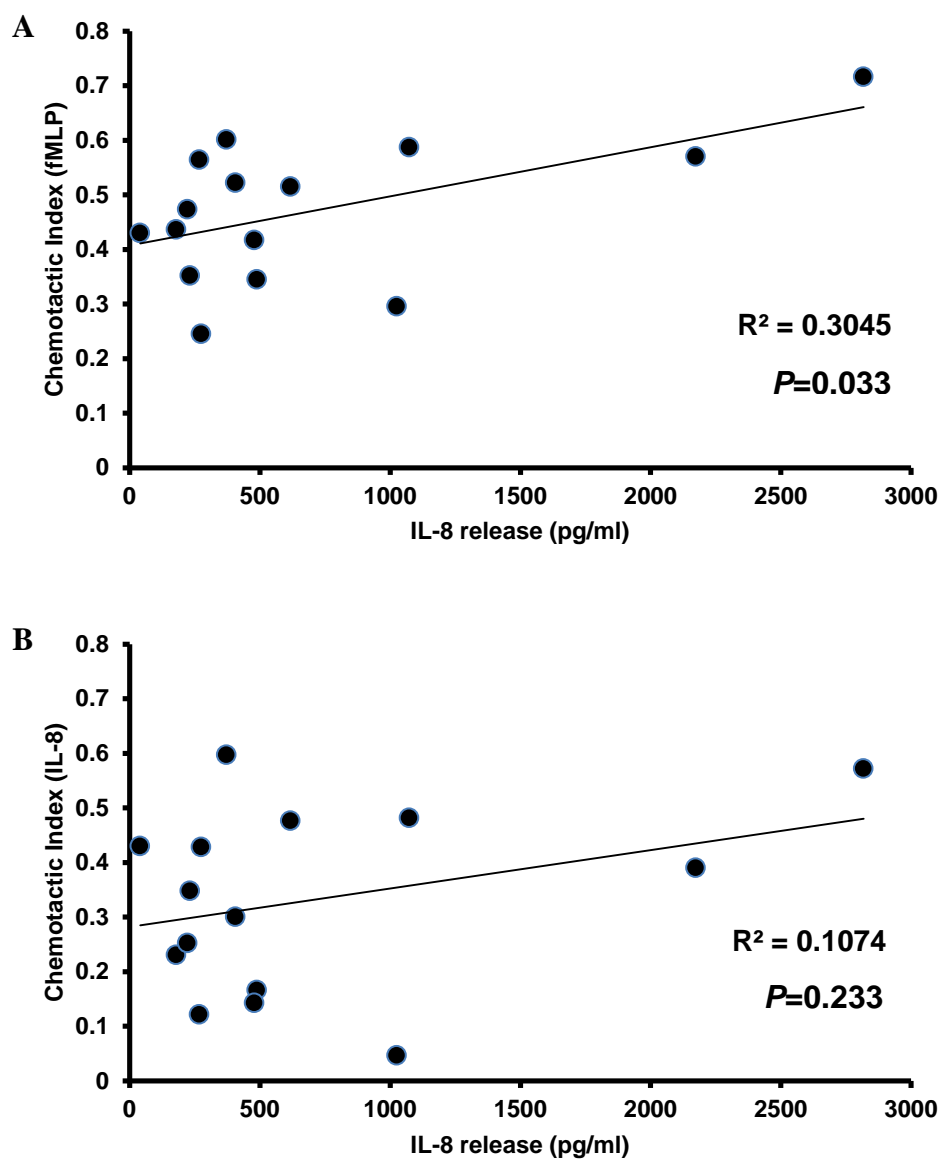


Figure 6.10. Correlation (n=16) between patient neutrophil IL-8 release (unstimulated; RPMI control) and chemotactic index in response to (A) fMLP and (B) IL-8.

## 6.4 DISCUSSION

### Neutrophil extracellular trap release in chronic periodontitis

The data presented in this chapter, generated using a recently developed fluorometric quantification assay (Palmer *et al.* 2012) has demonstrated, for the first time, a lack of significant differences in NET release from peripheral blood neutrophils from patients with chronic periodontitis compared to matched healthy controls. This adds to our current understanding of disease pathogenesis because, whilst ROS are essential for NET release (Brinkmann *et al.* 2004, Palmer *et al.* 2012), neutrophil hyper-active and –reactive ROS release (sections 1.6.3, Chapter 5) does not result in detectable differences in NET production.

Neutrophil extracellular trap release was initiated following stimulation with PMA (Brinkmann *et al.* 2004) and HOCl (Palmer *et al.* 2012), both of which are established NET stimuli. Initial studies quantified NETs using cell image counting but such methods are subject to observer bias (Brinkmann *et al.* 2004, Fuchs *et al.* 2007, Clark *et al.* 2007, Yost *et al.* 2009) and care is required because NET structures may be artefactual. For example, it has been questioned whether reliable discrimination between NETs and fibrin is possible using scanning electron microscopy analyses (Krautgartner *et al.* 2010). Fluorometric quantification of NETs was therefore used in this study to reduce the potential for bias and to obtain more objective data.

Neutrophil extracellular trap release is dependent upon neutrophil ROS generation and release (Brinkmann *et al.* 2004, Fuchs *et al.* 2007) and more specifically HOCl (Palmer *et al.* 2012). The first and subsequent NET studies predominantly used PMA to induce NET formation, with approximately one third of PMA-activated neutrophils isolated from human venous blood producing NETs *in vitro* (Brinkmann *et al.* 2004, Fuchs *et al.* 2007). Whilst PMA is non-physiological, it has the advantage that it causes direct activation of protein kinase C and generation of  $O_2^-$ , thus bypassing receptor-ligand binding on the neutrophil cell surface and subsequent phagocytosis. Data from this chapter has demonstrated that HOCl-stimulated neutrophils produce approximately 3 times more NETs compared to PMA, in agreement with the first study to demonstrate that NET release is regulated by HOCl (Palmer *et al.* 2012).

Given the requirement for ROS production to initiate NET release, it is interesting to note that, whilst the literature and the  $O_2^-$  data presented in this thesis supports neutrophil hyper-activity and -reactivity regarding ROS release in chronic periodontitis (sections 1.6.3, 5.2 & 5.3), there were no significant differences in NET release compared to healthy controls. One possible explanation may relate to the amount of ROS required, as it has been demonstrated that relatively large concentrations of HOCl ( $\geq 0.5\text{mM}$ ) are required to induce NET formation (Palmer *et al.* 2012). The lack of a significant difference in unstimulated NET release between patient and control neutrophils is most probably due to insufficient amounts of excess ROS associated with the ROS hyper-activity observed in chronic periodontitis to induce NET formation *per se*. This is supported by two observations. Firstly, hyperactivity in terms of ROS generation by unstimulated neutrophils appears to be limited to extracellular release rather than raised intracellular levels that would be required for NET release (Matthews *et al.* 2007). Secondly, the data demonstrate that 5nM PMA stimulates patient neutrophils to produce 5.6-7 times the amount of lucigenin detectable  $O_2^-$  than unstimulated cells (Table 5.2; Chapter 5) but 25nM PMA is required to induce consistent NET release. That there were no differences in PMA-stimulated NET release is not surprising given the fact that patient neutrophils did not exhibit hyper-reactivity to PMA compared to control neutrophils in terms of  $O_2^-$  production (Table 5.2 & Figure 5.2; Chapter 5).

While data using PMA appear to indicate that neutrophils from periodontitis patients do not differ from those isolated from control individuals, future studies, investigating NET formation in chronic periodontitis in response to physiologically-relevant bacterial stimuli associated with disease pathogenesis, are still required. Such studies might also include methods of investigating NET release involving multiple stimuli and/or longer, more relevant timescales associated with chronic inflammatory diseases such as periodontitis. For example, neutrophils from periodontitis patients exhibit ROS hyper-activity and –reactivity as well as hyper-reactivity in terms of IL-8 release following Fc $\gamma$ R and *F. nucleatum* stimulation (section 4.5.2), the combination of which may be relevant to disease pathogenesis given that IL-8 is also a recognised NET stimulant (Brinkmann *et al.* 2004).

Following stimulation, NET release requires large-scale decondensation and unfolding of nuclear chromatin by PAD-4 activation (Gyorgy *et al.* 2006, Wang *et al.* 2009). Interestingly,



whilst PAD-4 has been shown to localise to the tertiary granules of neutrophils (Theilgaard & Mönch 2005), *P. gingivalis* is currently the only recognised bacterial species to express a PAD enzyme (PPAD; Abdullah *et al.* 2013). There are currently no known published studies demonstrating bacterial PAD-induced NET release, however there is evidence that PPAD is important in autoantibody formation and the development and progression of rheumatoid arthritis (Wegner *et al.* 2010, Maresz *et al.* 2013). Given that *P. gingivalis* is a widely recognised periodontal pathogen and can survive and remain viable intra-cellularly (Li *et al.* 2008), it is possible that *P. gingivalis* may play an active role in histone hypercitrullination associated with NET formation (section 1.5.4.6) in the pathogenesis of chronic periodontitis.

Neutrophil extracellular trap release is relevant to chronic periodontitis pathogenesis since NETs have been visualised from gingival biopsy samples (Vitkov *et al.* 2009) and within purulent GCF exudates from patients with chronic periodontitis (Krautgartner & Vitkov 2008). In addition, transmission electron microscopic analyses of pocket epithelium biopsies from patients with chronic periodontitis have also shown the presence of NETs (Vitkov *et al.* 2010). Interestingly, it has been proposed that NETs can become incorporated as part of the biofilm structure (Hong *et al.* 2009). In addition, bacterial nucleic acids have been shown to form the framework for the bacterial community within plaque (Jakubovics & Kolenbrander 2010), although there are currently no known published studies investigating the role of NET DNA in the development of the dental plaque biofilm. Furthermore, in relation to the pathogenesis of chronic periodontitis, it is also important to appreciate that viable bacteria have defensive strategies to combat NET release, with many periodontal pathogens expressing extracellular DNases (Palmer *et al.* 2012) in order to evade NET entrapment. Unsurprisingly, bacteria expressing DNases have been shown to demonstrate increased NET survival *in vitro*, along with increased virulence and disease severity *in vivo* (Bieter *et al.* 2006, Buchanan *et al.* 2006, Berends *et al.* 2010). Further investigation as to the role of bacterial and host-derived DNases will therefore add to our current understanding of the role of NETs in the pathogenesis of chronic periodontitis.

Neutrophil extracellular trap release is an important neutrophil defence strategy that has been shown to occur in a number of systemic diseases associated with chronic periodontitis including rheumatoid arthritis (de Pablo *et al.* 2009, Routsias *et al.* 2011, Nesse *et al.* 2012,

Rohrbach *et al.* 2012, Thomas *et al.* 2012), renal disease (Palic *et al.* 2007, Kessenbrock *et al.* 2009), respiratory disease (Aulik *et al.* 2010, Hemmers *et al.* 2011, Saffarzadeh *et al.* 2012), systemic lupus erythematosus (Garcia-Romo *et al.* 2011), cerebrovascular inflammation (Allen *et al.* 2012) and atherosclerosis (Doring *et al.* 2012). The formation of NETs may have the potential to further increase the likelihood of collateral host tissue damage in the pathogenesis of chronic periodontitis along with increasing the risk of co-morbidity. For example NETs have been shown to release cytokines (IL-1 $\beta$ , IL-8 and TNF- $\alpha$ ) from platelets and the THP-1 monocyte cell line thereby providing evidence of NETs augmenting local cytokine networks within tissues (Keshari *et al.* 2012). It is therefore possible that NET release may have the potential to affect the co-ordination of different inflammatory-immune responses and therefore the risk of co-morbidity associated with the pathogenesis of chronic periodontitis. Furthermore it may be possible that by their very nature as “traps”, local NET release may impede other important neutrophil functions such as the diffusion of cytokines and also neutrophil chemotaxis. Ineffective removal or delayed neutrophil tissue transit times will increase the likelihood of collateral host tissue damage, irrespective of there being no detectable differences in NET release *in vitro*, especially given the hyper-inflammatory neutrophil ROS and cytokine phenotype observed in chronic periodontitis.

### **Neutrophil chemotactic accuracy in chronic periodontitis**

Compared to healthy controls, the chemotaxis data presented in this chapter demonstrate significantly impaired neutrophil directional chemotactic accuracy (chemotactic index), together with reduced velocity and speed of migration towards fMLP. While the medians for these parameters were also lower for patient cells using IL-8 as chemoattractant, the differences compared to control cells were not statistically significant using 2-tailed Mann-Whitney analyses. These data were generated using a novel methodology recently developed with colleagues at the University of Glasgow, and more accurately represents chemotaxis rather than chemokinesis by depicting individual cell movement in relation to chemoattractant sources representative of the complexity of neutrophil migration in the pathogenesis of chronic periodontitis.

The chemotaxis chamber used in the studies reported in this thesis was recently developed as an improved chamber for direct visualisation and characterisation of the complex cell migratory dynamics representative of chemotaxis (Muinonen-Martin *et al.* 2010). Previous studies investigating “chemotaxis” have used various transwell methods with filters (typically 5µm for neutrophil studies) including Boyden chambers (Van Dyke *et al.* 1980, McMullen *et al.* 1981, Coles *et al.* 1989, Kinane *et al.* 1989, Champagne *et al.* 1998, Meyle & Axmann-Kremar 1999, Shibata *et al.* 2001, Dias *et al.* 2008, Yagi *et al.* 2009, Mizuno *et al.* 2011), Wilkinson’s method (Bhansali *et al.* 2013) along with *in vivo* external skin window testing (Palmer *et al.* 1993). Whilst these methods are useful, they only indirectly measure unidirectional movement of cells, which is more representative of chemokinesis (i.e. the random migration of cells observed in a homogenous solution of an extracellular signal; Wells 2000). In contrast, direct visualisation chambers have the advantage of allowing individual, real-time, cell migration to be observed using time-lapse microscopy which is more representative of chemotaxis (i.e. the process by which the direction of motile cells is biased along a concentration gradient of soluble factors and extracellular signals; Insall 2010).

There are many examples of direct visualisation chemotaxis chambers including the Zigmond chamber (Zigmond 1977), the Dunn chamber (Zicha *et al.* 1991), agarose (Kinane *et al.* 1989, Mouynet *et al.* 1994, Ibarra *et al.* 2006, Srinivas *et al.* 2012), pipette (Gerisch & Keller 1981) and microfluidic chamber assays (Li Jeon *et al.* 2002). The new direct visualisation chemotaxis chamber used in this thesis (section 2.2.10; Muinonen-Martin *et al.* 2010) is essentially a refined Dunn chemotaxis chamber with a number of published advantages including ease of handling, chemoattractant gradients with defined directions along with the ability to use #1.5 cover slips thereby allowing the use of high numerical aperture oil immersion lenses (Muinonen-Martin *et al.* 2010). The concurrent use of time-lapse phase contrast microscopy also allows tracking of individual cell migration pathways. The chemotactic accuracy data presented in this chapter was generated using novel methodology providing both qualitative and quantitative measurements of neutrophil chemotaxis in chronic periodontitis compared to healthy controls using a novel direct visualisation chemotaxis chamber.

In terms of periodontitis, the vast majority of studies investigating chemotaxis (or more accurately chemokinesis) have been conducted with juvenile/aggressive periodontitis patients (reviewed by Ryder 2010), which is relatively uncommon in comparison to chronic periodontitis. To date there are only 8 studies investigating peripheral blood neutrophil chemokinesis in chronic periodontitis (Van Dyke *et al.* 1980, McMullen *et al.* 1981, Altman *et al.* 1985, Zafiropoulos *et al.* 1991, Mouynet *et al.* 1994, Kumar & Prakash 2012, Srinivas *et al.* 2012; Table 1.3). The data presented in this chapter demonstrates impaired directional chemotaxis, speed and velocity of movement in patients with chronic periodontitis compared to controls in agreement with previous literature demonstrating impaired chemokinesis in chronic periodontitis (Van Dyke *et al.* 1980, Zafiropoulos *et al.* 1991, Kumar & Prakash 2012). While reduced chemotactic index, speed and velocity of movement of patient cells compared to control neutrophils was only significant in the presence of fMLP, median values for these parameters were also lower for patient cells in response to IL-8. This lack of significance for the IL-8 data may result from the effectiveness of each chemoattractant, as fMLP caused significantly greater neutrophil migration and larger differences between patient and control cells compared to IL-8. This is particularly pertinent as, assuming that patient cells would have defective chemotactic accuracy relative to healthy control cells, 1-tailed Mann-Whitney analysis of the IL-8 data shows significant or nearly significant differences between patient and control cells for chemotactic index ( $P=0.0424$ ), speed ( $P=0.055$ ) and velocity ( $P=0.0484$ ). Although it could be argued that the 1-tailed assumption is acceptable, based on data from the current literature, further assays on larger numbers of cell samples are required to confirm that the chemotactic accuracy of patient cells to IL-8 is statistically lower than that of cells from healthy controls at the 2-tailed level.

Interestingly, reduced neutrophil chemokinesis has also been shown in patients with chronic periodontitis with and without diabetes mellitus (McMullen *et al.* 1981) as well as smokers compared to non-smokers with the same periodontal status (Srinivas *et al.* 2012). Whilst defective chemokinesis is recognised in the pathogenesis of chronic periodontitis, the data presented in this chapter is the first study to observe and directly measure individual cell migratory patterns more accurately representative of directional chemotactic accuracy in the pathogenesis of chronic periodontitis. Furthermore, these data highlight the complexity of neutrophil directional migratory patterns compared to data collected using indirect

unidirectional assays. In addition, the use of time-lapse videomicroscopy and cell-tracking allowed the calculation of overall neutrophil directional chemotactic accuracy, speed and velocity of movement in relation to individual cell migratory pathways.

The process of neutrophil recruitment to sites of periodontal inflammation initially involves endothelial transmigration via diapedesis, along with local generation of chemotactic signals (e.g. IL-8, fMLP) which then facilitate neutrophil migration towards sites of infection. Neutrophil chemotaxis involves several stages which to date are relatively poorly understood. Bacterial formylpeptides are one of the major chemotactic stimuli (Maney & Walters 2009) and neutrophils typically express approximately 55,000 G-protein-coupled formylpeptide receptors (FPRs) per cell (Pike & Snyderman 1988, Maney & Walters 2009), which when activated initiate a cascade of enzymatic reactions resulting in cell polarisation, orientation and migration towards the source of the chemoattractant (Snyderman & Goetzl 1981, Springer 1995, Katanaev 2001, Yagi *et al.* 2009). Differences in chemotactic cell surface receptors/co-receptors for chemotactic stimulants such as glycoprotein 110 or CD38 (Van Dyke *et al.* 1990) or single nucleotide polymorphisms of FPRs (Perez *et al.* 1991, Jones *et al.* 2003, Maney & Walters 2009) or a reduced number of FPR receptors (Meyle & Axmann-Kremar 1999), and/or subsequent pseudopodia generation may offer an explanation for the impaired chemotactic accuracy, speed and velocity of movement in periodontitis neutrophils presented in this chapter. A defect in pseudopodia activity could result in morphological differences and subsequent ability of neutrophils to migrate and their directional chemotactic accuracy. The videomicroscopy images of migrating neutrophils, collected in this study, have since been used to undertake preliminary elliptical factor analyses by measuring the greatest distance between the cell membranes and the perpendicular distance of migrating neutrophils. These analyses have revealed no significant neutrophil shape differences during chemotaxis towards fMLP and IL-8 (Appendix III). Interestingly another factor to consider relates to *in vitro* methodology, since neutrophils have been shown to exhibit a reduced number of FPR receptors when measured at 22°C (Coles *et al.* 1989). This is important given that studies of neutrophil migration do not appear to control or state the temperature with which experiments were performed. All of the chemotaxis assays performed in this chapter were performed at 18.2-23.2°C rather than at an *in vivo* body temperature of ~37°C. However both patient and control neutrophils were assayed at the same temperature and therefore any differences due to

temperature would need to have differentially affected patient cells if this was to contribute to the observed defect in neutrophil directional chemotactic accuracy.

With regards to the pathogenesis of periodontitis, it is interesting that data presented in this thesis revealed significantly increased cytokine release from periodontitis neutrophils when comparing combined data for all stimuli along with several individual stimulatory conditions (Tables 4.2, 4.3). It has previously been hypothesised that the biological basis for altered neutrophil chemotaxis may not be solely due to an intrinsic cellular defect, but may also in part be due to a quantitatively small but biologically significant elevation in serum cytokine concentrations (Meyle 1993, Agarwal *et al.* 1994). This is especially true with regards to the increased neutrophil IL-8 cytokine release presented in this thesis (section 4.5.2), which is a widely known chemoattractant and was therefore included as a positive chemoattractant within the chemotaxis assay methodology. It was thought that it may be possible that neutrophil IL-8 release could effectively result in auto-recruitment and auto-migratory patterns resulting in defective directional chemotaxis in periodontitis neutrophils. However a moderate positive correlation between neutrophil IL-8 release and chemotactic index towards fMLP was observed suggestive that neutrophil IL-8 release may in fact, albeit minimally, assist with neutrophil chemotaxis towards bacterial-derived chemoattractants. Another factor to consider is that neutrophil activation and subsequent IL-8 release is more likely to occur when neutrophils are already at sites of bacterial infiltration and inflammation and is therefore more likely to affect surrounding neutrophil chemotaxis through cytokine diffusion rather than individual neutrophil IL-8 release and chemotaxis *per se*. That the correlation was more noticeable for fMLP, compared to IL-8 as chemoattractants, may possibly relate to the greater chemotactic potential of fMLP, with greater chemotactic index, speed and velocity in the presence of fMLP compared to IL-8 presented in this chapter. As such, it is possible that neutrophils predominantly migrate towards bacterial components such as fMLP rather than host-derived factors, with migrating neutrophils concurrently releasing IL-8 which may additionally assist with the overall neutrophil migratory pattern towards the chemoattractant source as part of the inflammatory-immune response. Interestingly *P. gingivalis* has also been shown to secrete degradative enzymes (gingipains) that significantly enhance the chemotactic activity of IL-8<sub>77aa</sub> released from non-immune cells, but decrease the chemotactic activity of IL-8<sub>72aa</sub> released from immune cells, thereby suggesting that during periodontitis secreted

gingipains may differentially affect neutrophil chemotaxis in response to IL-8 depending on the cellular source of the cytokine (Dias *et al.* 2008), and this is likely to be important in the pathogenesis of chronic periodontitis.

Overall, the NET and chemotaxis data presented in this chapter generated using recently developed assays add to our current understanding of the pathogenesis of chronic periodontitis. This is the first study to quantify NET release, with a lack of detectable differences in unstimulated or PMA- and HOCl-stimulated NET release between patient and control neutrophils. In addition, these data more accurately depict reduced chemotactic accuracy of peripheral blood neutrophils in chronic periodontitis. Reduced chemotactic accuracy of a hyper-inflammatory neutrophil phenotype will increase the likelihood of collateral host tissue damage associated with the pathogenesis of chronic periodontitis.

# **CHAPTER 7**

## **RESULTS & DISCUSSION**

**C-reactive protein characterisation & effects of buffer  
constituents on chemiluminescence and the neutrophil respiratory  
burst**



## 7.1 INTRODUCTION

C-reactive protein (CRP) from human plasma was supplied commercially (Sigma C4063) as pentameric CRP in 20mM Tris, pH 7.8-8.2, containing 280mM sodium chloride, 5mM calcium chloride and 0.1% sodium azide as a preservative. The product specification stated a  $\geq 90\%$  purity determined by SDS electrophoresis. Prior to determining the effects of CRP on neutrophil ROS generation and release (Chapter 8) the commercial CRP samples were independently characterised to ascertain molecular form and the presence of contaminants (section 7.2). The effects of CRP buffer constituents upon cell-free chemiluminescence signals (section 7.4) and neutrophil ROS generation (section 7.5) were also measured. In addition, the antioxidant capacity of CRP was determined for the first time (section 7.7) as this could influence subsequent data interpretation of the effects of CRP upon *ex-vivo* neutrophil ROS generation and release presented in Chapter 8. Furthermore, the molecular form of CRP was determined to confirm the presence of soluble pentameric CRP rather than altered or modified CRP (mCRP). The latter is insoluble, antigenically distinct and naturally expressed at vascular sites, also having been associated with vascular damage and cardiovascular disease (Diehl *et al.* 2000).

## 7.2 C-REACTIVE PROTEIN CHARACTERISATION

The purity and molecular weight of the commercial CRP samples were determined using liquid chromatography tandem mass spectrometry (section 2.2.11.3) and analytical ultracentrifugation (AUC; section 2.2.11.4).

### **7.2.1 Liquid chromatography tandem mass spectrometry**

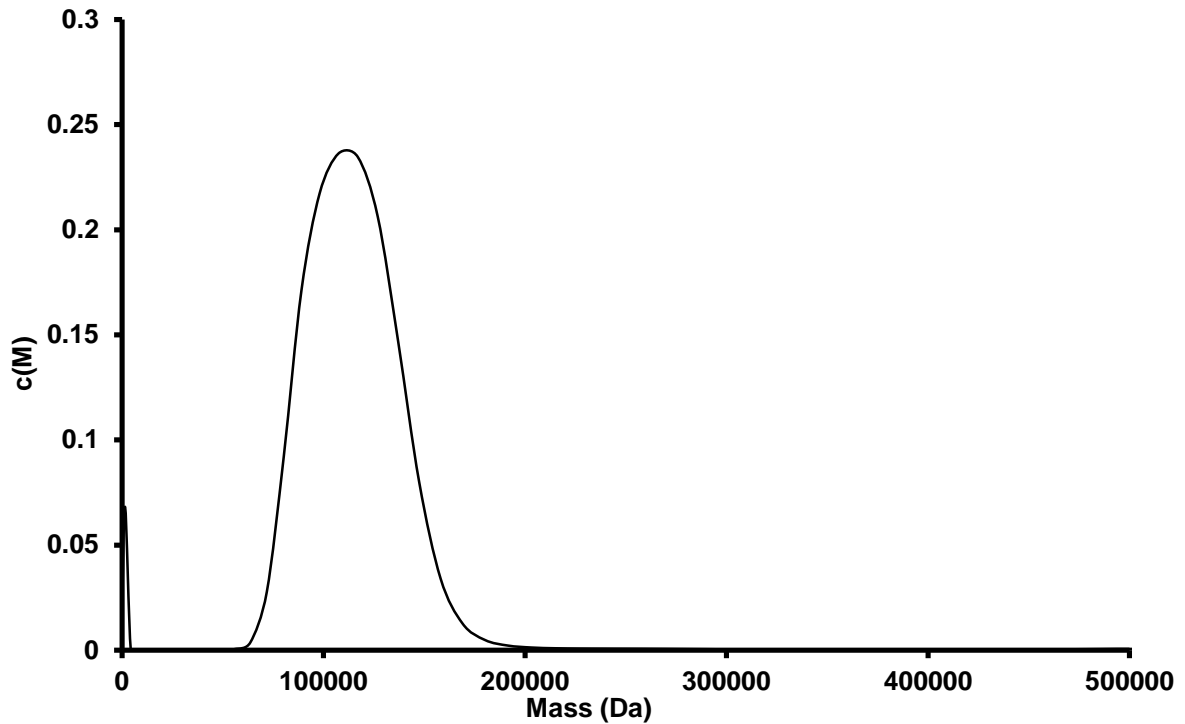
Analysis of protein peptide sequences confirmed the predominant presence of isoform 1 CRP (>98%) plus 21 microcontaminant proteins, 11 of which were derived from epidermis or saliva (Table 7.1). These latter components are commonly present in proteins purified from biological samples due to the extensive laboratory processes required for purification. Isoform 1 of CRP was characterised by 23 peptides, which were consistent with being derived from monomeric CRP units of molecular weight 25,023Da containing 224 amino acids. The intensity of the peptide precursor ions identified from the CRP samples were compared to those of peptide ions from contaminants. Contaminant intensity was <2% of the CRP signal, based upon intensity analyses of the major peptides, demonstrating CRP purity was >98%

Accession #	Coverage	# Peptides	#AAs	MW [Da]	Score	Description
IPI:IPI00022389.1	29.02	23	224	25023	621.96	Isoform 1 of C-reactive protein
IPI:IPI00009865.4	23.29	12	584	58792	465.21	Keratin, type I cytoskeletal 10 *
IPI:IPI00220327.4	17.24	11	644	65999	376.98	Keratin, type II cytoskeletal 1 *
IPI:CON_00745872.2	12.15	7	609	69321	245.90	Isoform 1 of Serum albumin precursor *
IPI:CON_00021304.1	5.43	3	645	65825	142.01	Keratin, type II cytoskeletal 2 epidermal *
IPI:IPI00853045.1	15.81	2	234	25682	135.41	Anti-RhD monoclonal T125 kappa light chain
IPI:CON_00019359.3	4.65	2	623	62092	122.33	Keratin, type I cytoskeletal 9 *
PROT:P00761	16.45	4	231	24394	119.36	Trypsin - Sus scrofa (Pig). - [TRYP_PIG]
IPI:CON_00714876.1	5.45	3	606	63127	105.07	ENSEMBL:ENSBTAP00000038253 (Bos taurus) 63 kDa protein
IPI:IPI00922744.1	4.24	1	354	38114	100.77	Complement protein C4B frameshift mutant (Fragment) *
IPI:IPI00646265.1	7.08	1	226	25554	99.59	Amylase, alpha 1B *
IPI:IPI00830047.2	17.99	2	139	14669	75.34	IGL@ protein
IPI:IPI00789295.1	14.71	1	102	11154	71.91	11 kDa protein
IPI:IPI00293665.9	3.37	2	564	60030	70.89	Keratin, type II cytoskeletal 6B *
IPI:IPI00431645.1	4.27	1	281	31362	61.95	HP protein
IPI:IPI00442911.1	6.73	1	208	22212	59.23	CDNA FLJ26266 fis, clone DMC05613
IPI:IPI00382804.1	4.85	1	227	24181	56.25	EEF1A protein (Fragment)
IPI:IPI00397801.4	0.50	1	2391	247928	47.81	Filaggrin-2 *
IPI:IPI00871949.1	4.97	1	965	102093	43.85	PREDICTED: filaggrin variant *
IPI:IPI00027547.2	10.00	1	110	11277	42.70	Dermcidin *
IPI:IPI00791348.1	5.73	1	192	19583	42.22	20 kDa protein
IPI:IPI00514530.5	5.54	1	289	32254	41.59	Putative uncharacterized protein ACTA1

**Table 7.1. Liquid chromatography tandem mass spectrometry MS/MS analysis of commercial CRP sample (Sigma C4063).** Protein peptide sequences confirm the predominant presence (>98%) of isoform 1 CRP plus 21 microcontaminant proteins, 11 of which are derived from epidermis or saliva (\*).

### 7.2.2 Analytical ultracentrifugation

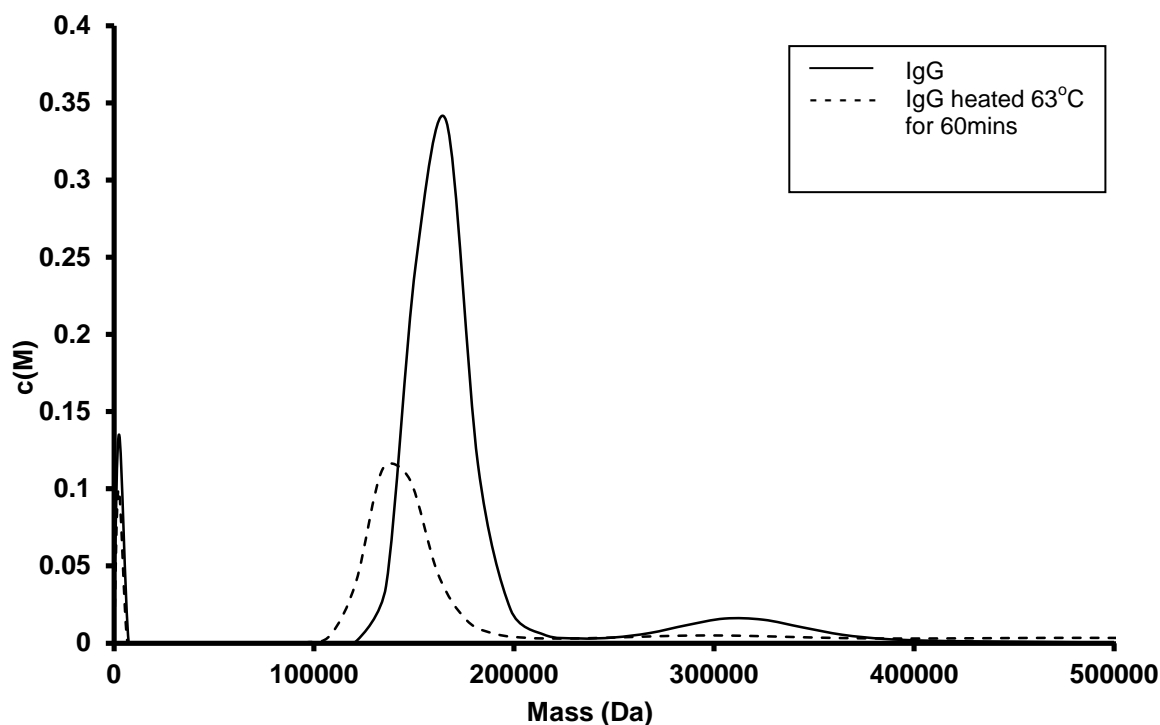
Analytical ultracentrifugation analysis of the commercial CRP sample, under non-dissociating conditions, revealed a sedimentation coefficient of ~6.2S consistent with a molecular mass of ~117kDa and corresponding to native, pentameric CRP (Okemefuna *et al.* 2010; Figure 7.1).



**Figure 7.1. A SEDFIT  $c(M)$  deconvolution of sedimentation velocity analytical ultracentrifugation data collected on a dialysed and filtered commercial CRP sample.** Please note the presence of 1 peak corresponding to ~117kDa consistent with pentameric CRP. Data was collected in triplicate on a Beckman XL-I proteome lab at 20,000rpm with particle migration monitored by scanning the cells at 280nm.

### **7.3 ANALYTICAL ULTRACENTRIFUGATION OF UNHEATED AND HEATED IgG SAMPLES**

The formation of heat-aggregated IgG (section 2.2.5.1) was confirmed by analytical ultracentrifugation prior to its inclusion as a stimulus in the ROS assays that aimed to investigate the effects of CRP on the neutrophil respiratory burst presented in Chapter 8. Analytical ultracentrifugation of unheated IgG samples (freeze-dried IgG purified from human serum (Sigma I4506), reconstituted in 150mM NaCl) revealed two bands with sedimentation coefficients of ~6.5S (166kDa; IgG monomer) and 9.8S (300kDa; IgG dimer) corresponding to ~89% and ~11% of the protein analysed. Heated IgG (63°C for 60 minutes) also gave two bands with sedimentation coefficients of ~6.1S (134kDa; monomer-like) and ~10S (300kDa; dimer) but these only accounted for ~35% and ~5% of the protein sedimented. Approximately 60% of the heated IgG sample failed to sediment indicating a molecular weight of >500kDa consistent with the presence of IgG aggregates (Figure 7.2).



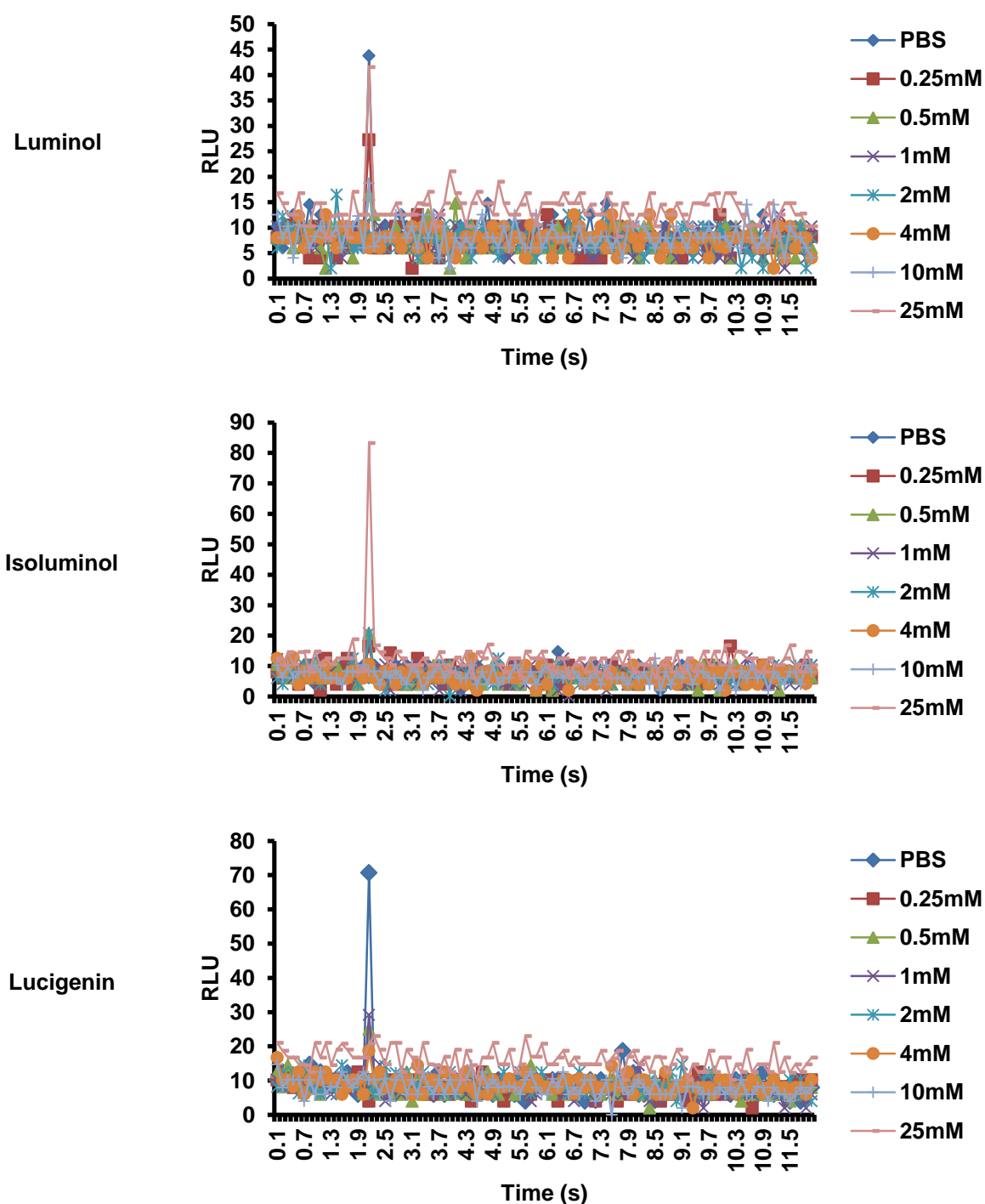
**Figure 7.2. A SEDFIT  $c(M)$  deconvolution of sedimentation velocity analytical ultracentrifugation data collected on unheated and heated IgG samples.** Unheated IgG revealed 2 peaks ( $\sim 6.5S$ ; 166kDa; monomer &  $9.8S$ ; 300kDa; dimer) corresponding to  $\sim 100\%$  of the protein analysed. Heated IgG also gave 2 peaks ( $\sim 6.1S$ ; 134kDa; monomer-like &  $\sim 10S$ ; 300kDa; dimer) that accounted for  $\sim 40\%$  of the protein sedimented. Approximately 60% of the heated IgG sample failed to sediment indicating a molecular weight of  $>500kDa$ . Data was collected in triplicate on a Beckman XL-I proteome lab at 20,000rpm with particle migration monitored by scanning the cells at 280nm.

## **7.4 EFFECT OF TRIS BUFFER ON LUMINOL, ISOLUMINOL & LUCIGENIN-ENHANCED CHEMILUMINESCENCE**

The ability of Tris buffer, equivalent to that in which the commercial CRP was supplied and stored but without sodium azide, -to directly cause chemiluminescence (section 2.2.11.5), or alter the chemiluminescence signal generated following addition of H<sub>2</sub>O<sub>2</sub>, was determined for luminol, isoluminol and lucigenin test systems (section 2.2.11.6), as described below.

### **7.4.1 Direct effect of Tris buffer on luminol, isoluminol and lucigenin-enhanced chemiluminescence**

There were no discernable direct effects of Tris buffer on luminol, isoluminol or lucigenin-enhanced chemiluminescence at the concentrations tested (Figure 7.3), other than occasional peaks of light output resulting from the initial minor environmental disturbance due to the injection of Tris buffer.



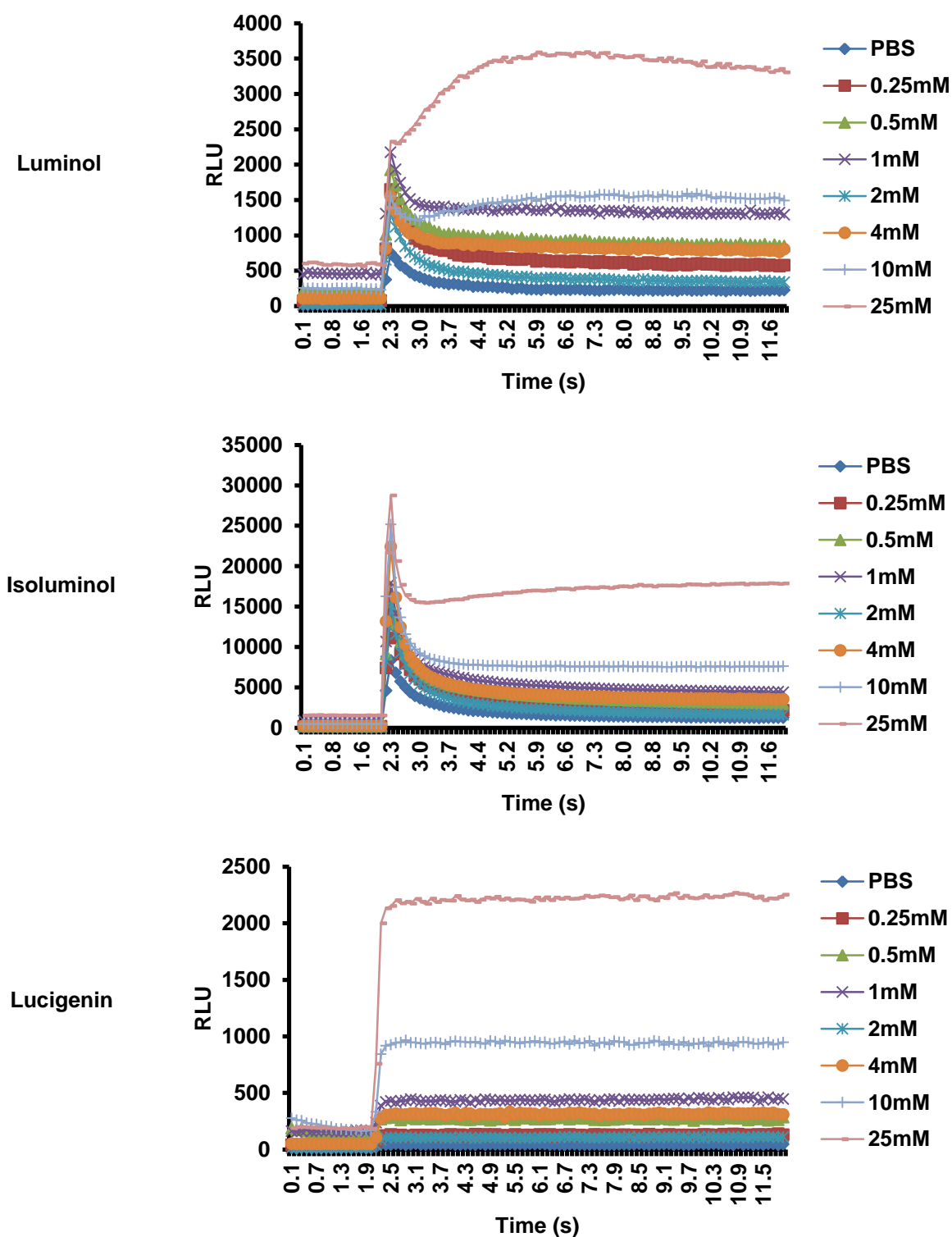
**Figures 7.3. Lack of a direct effect of 0.25, 0.5, 1, 2, 4, 10 & 25mM Tris buffer on luminol, isoluminol and lucigenin chemiluminescence (cell-free system).** Light output was monitored before (2s), during and after (10s) automatic injection of freshly prepared Tris buffer diluted in distilled water. Data presented as mean relative light units (RLU) and is representative of a single experiment performed in quadruplicate. Please note the occasional “peak” in light output at 2s due to the minor environmental disturbance caused by the injection of Tris buffer.



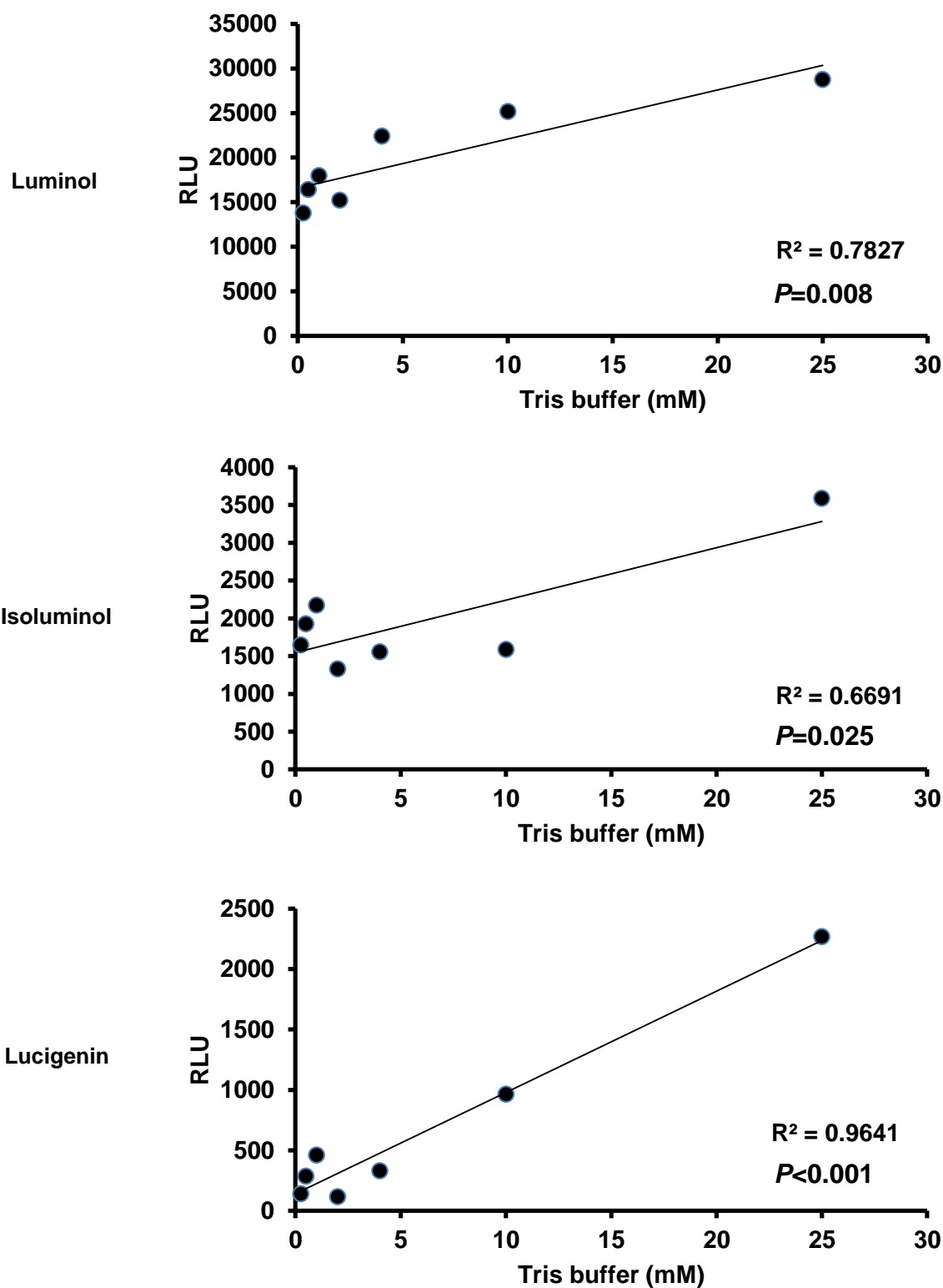
#### **7.4.2 Effect of Tris buffer on H<sub>2</sub>O<sub>2</sub>-induced luminol, isoluminol and lucigenin-enhanced chemiluminescence**

The presence of Tris buffer resulted in significant changes in H<sub>2</sub>O<sub>2</sub>-induced luminol, isoluminol and lucigenin-enhanced chemiluminescence (Friedman  $P < 0.0001$ ). Addition of all tested concentrations of Tris buffer (0.25-25mM) resulted in significant increases in luminol, isoluminol and lucigenin enhanced chemiluminescence (Dunn's multiple comparison's test  $P < 0.05$ ; Figure 7.4). The one exception to this was 2mM Tris, which had no significant effect on isoluminol and lucigenin chemiluminescence and had less effect on luminol light emission than 0.25mM Tris, suggesting that there may have been an error in its preparation. Moderate and strong significant positive correlations were observed between Tris buffer concentration (including the 2mM Tris data) and H<sub>2</sub>O<sub>2</sub>-induced chemiluminescence (Figure 7.5).

The addition of 1mM Tris, equivalent to that used in the enhanced chemiluminescence assays to determine the effects of CRP on neutrophil ROS generation and release (section 2.2.11.8; Chapter 8), resulted in significant increases in chemiluminescence compared to PBS control in the presence of luminol (17,957 & 8,343RLU; 215.2%;  $P < 0.001$ ), isoluminol (2,172 & 750RLU; 289.6%,  $P < 0.001$ ) and lucigenin (463 & 54RLU; 857.4%,  $P < 0.001$ ).



**Figure 7.4. Enhancement of hydrogen peroxide induced light output caused by the presence of 0.25, 0.5, 1, 2, 4, 10 and 25mM Tris buffer using luminol, isoluminol and lucigenin chemiluminescence substrates (cell-free system).** Light output was monitored before (2s), during and after (10s) automatic injection of freshly prepared  $\text{H}_2\text{O}_2$  solution. Data presented as mean relative light units (RLU) and representative of a single experiment performed in sextuplicate.



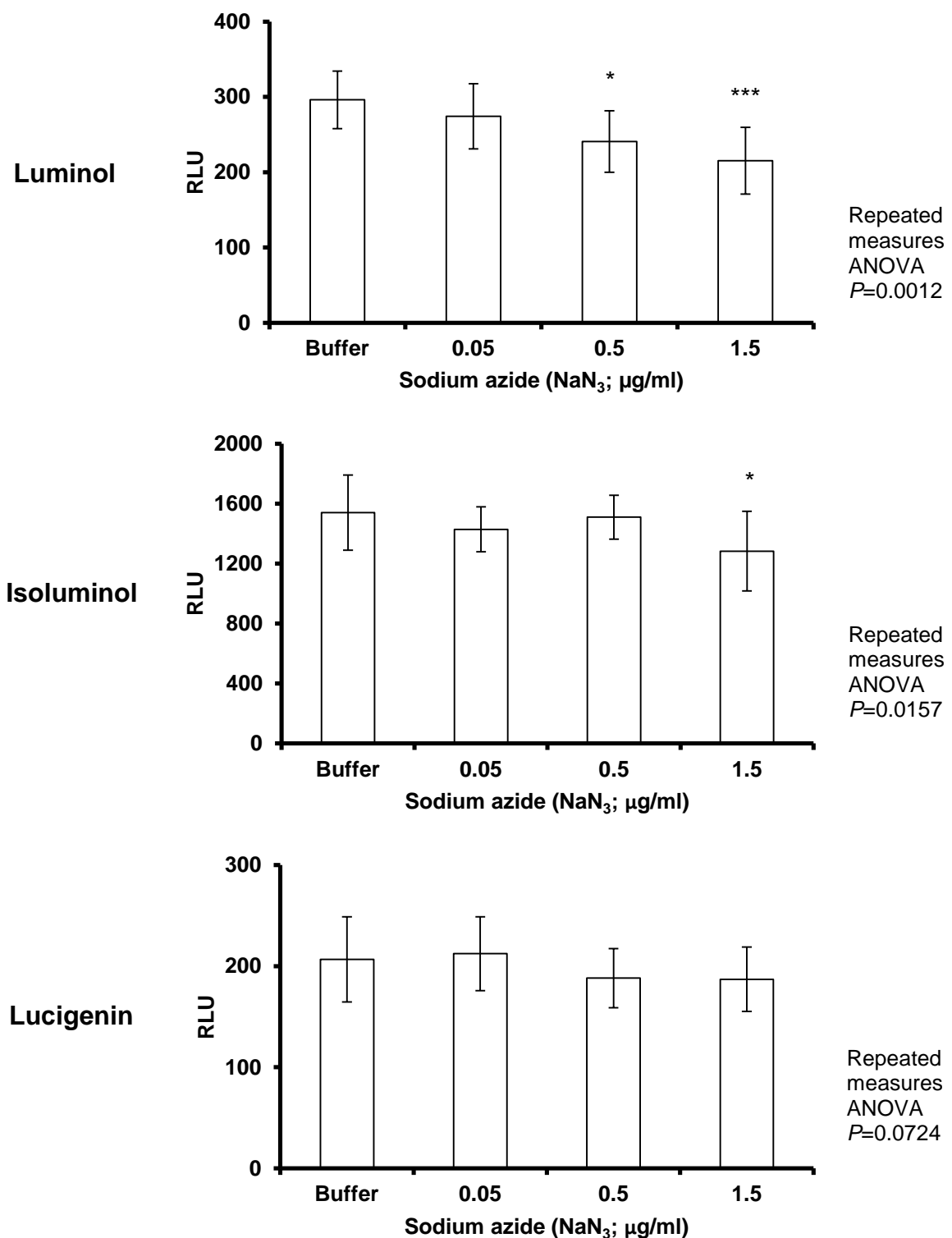
**Figure 7.5. Correlations between Tris buffer concentration and maximal  $H_2O_2$ -induced luminol, isoluminol and lucigenin enhanced chemiluminescence.** Data representative of a single experiment performed in sextuplicate.

## **7.5 EFFECT OF SODIUM AZIDE ON NEUTROPHIL ROS GENERATION**

The commercial CRP contained 0.1% (1mg/ml) sodium azide ( $\text{NaN}_3$ ) as a preservative, which is known to inhibit MPO, catalase and HRP activities (Ortiz de Montellano *et al.* 1988, VanUffelen *et al.* 1998). Concentrations of sodium azide (0.05, 0.5 and 1.5 $\mu\text{g/ml}$ ) equivalent to those that would be present in the neutrophil enhanced-chemiluminescence assays when examining the effects of 0.1, 1 and 3 $\mu\text{g/ml}$  CRP were analysed to determine their potential effects on neutrophil ROS generation and release, as described below.

### **7.5.1 Effect of sodium azide on unstimulated neutrophil ROS generation**

Addition of sodium azide significantly altered neutrophil ROS generation determined by luminol ( $P=0.0012$ ) and isoluminol ( $P=0.0157$ ) chemiluminescence that detect predominantly intra and extracellular HOCl respectively (Figure 7.6). 1.5 $\mu\text{g/ml}$  and 0.5 $\mu\text{g/ml}$  sodium azide significantly reduced neutrophil ROS generation detected by luminol compared to vehicle control ( $P<0.05$ ). In addition, 1.5 $\mu\text{g/ml}$  sodium azide significantly reduced neutrophil extracellular ROS detected by isoluminol ( $P<0.05$ ). In contrast, addition of sodium azide did not significantly affect neutrophil extracellular  $\text{O}_2^-$  detected by lucigenin at the concentrations tested ( $P=0.07$ ).



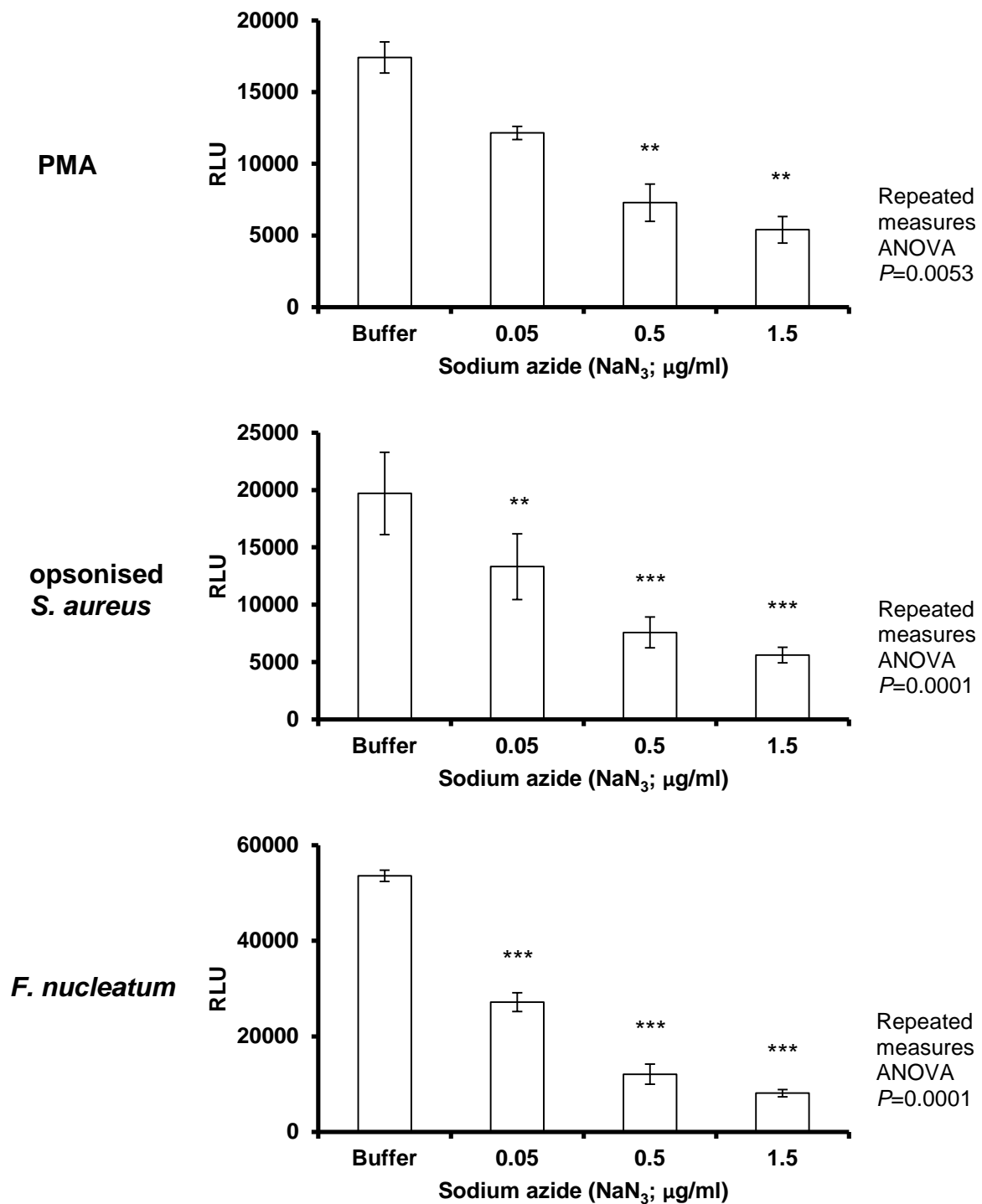
**Figure 7.6. Effect of sodium azide on unstimulated neutrophil ROS generation detected by luminol, isoluminol and lucigenin enhanced chemiluminescence.** Data presented as mean peak relative light units (RLU)  $\pm$  standard deviation, and representative of a single experiment performed in octuplicate.  $P$ -values for comparisons with buffer/ $0\mu\text{g/ml}$  azide-treated cells determined by Bonferroni multiple comparisons test, \* $P<0.05$ , \*\*\* $P<0.001$ .

### 7.5.2 Effect of sodium azide on stimulated neutrophil ROS generation

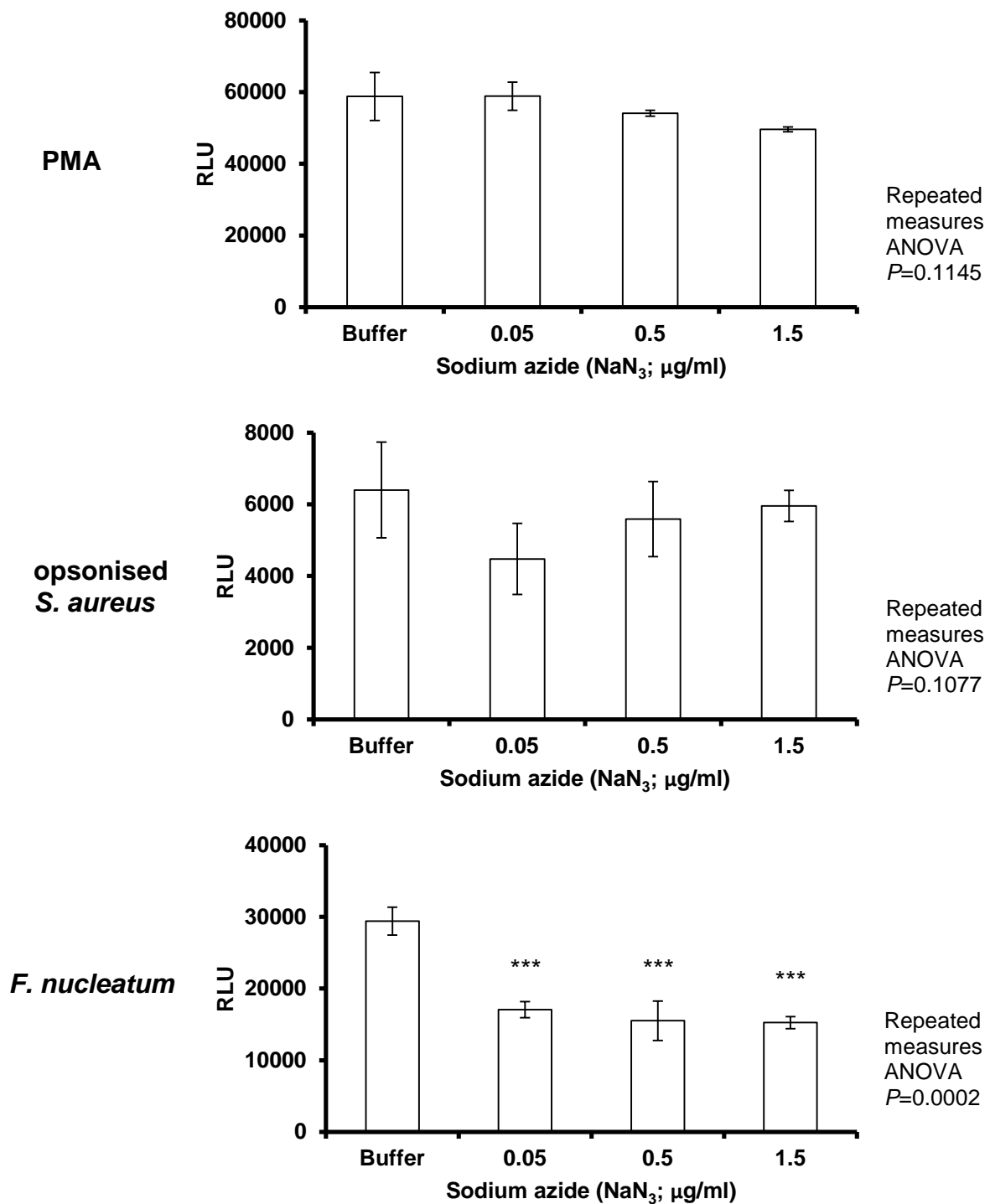
Neutrophil stimulation was performed following pre-incubation and in the presence of sodium azide. Data is representative of a single experiment performed in triplicate for opsonised *S. aureus* and *F. nucleatum*, and in duplicate for PMA stimulation. Given the obvious effects, despite the limitations of the data, indicative statistical analyses were performed.

Sodium azide significantly reduced, in a dose-dependent manner, PMA, FcγR and TLR stimulated neutrophil ROS generation detected by luminol chemiluminescence detecting predominantly intracellular HOCl (Figure 7.7). Mean levels of luminol chemiluminescence following FcγR and TLR stimulation was significantly lower in the presence of all tested concentrations of sodium azide (0.05-1.5μg/ml;  $P<0.01$ ) with 71.5% and 84.8% reductions in the presence of 1.5μg/ml sodium azide respectively. Similarly, 0.5μg/ml and 1.5μg/ml sodium azide significantly reduced PMA stimulated luminol-detected intracellular HOCl generation by 58.2% and 69.0% respectively ( $P<0.01$ ).

There were no significant differences in FcγR stimulated neutrophil extracellular HOCl release detected by isoluminol in the presence of sodium azide at the concentrations tested (0.05-1.5μg/ml; Figure 7.8). Furthermore, whilst the mean PMA stimulated neutrophil extracellular ROS detected by isoluminol and lucigenin was reduced in a dose-dependent manner in the presence of sodium azide, this was not statistically significant (Figures 7.8 & 7.9). *Fusobacterium nucleatum* stimulated isoluminol-detectable extracellular HOCl showed a mean decrease of 45.8% due to sodium azide at all concentrations tested ( $P<0.001$  for 0.05-1.5μg/ml). In contrast, FcγR and TLR stimulated extracellular O<sub>2</sub><sup>-</sup> release detected by lucigenin significantly differed in the presence of sodium azide, plateauing with 82.8% and 55.8% increases in the presence of 0.5μg/ml sodium azide respectively ( $P<0.001$ ; Figure 7.9).

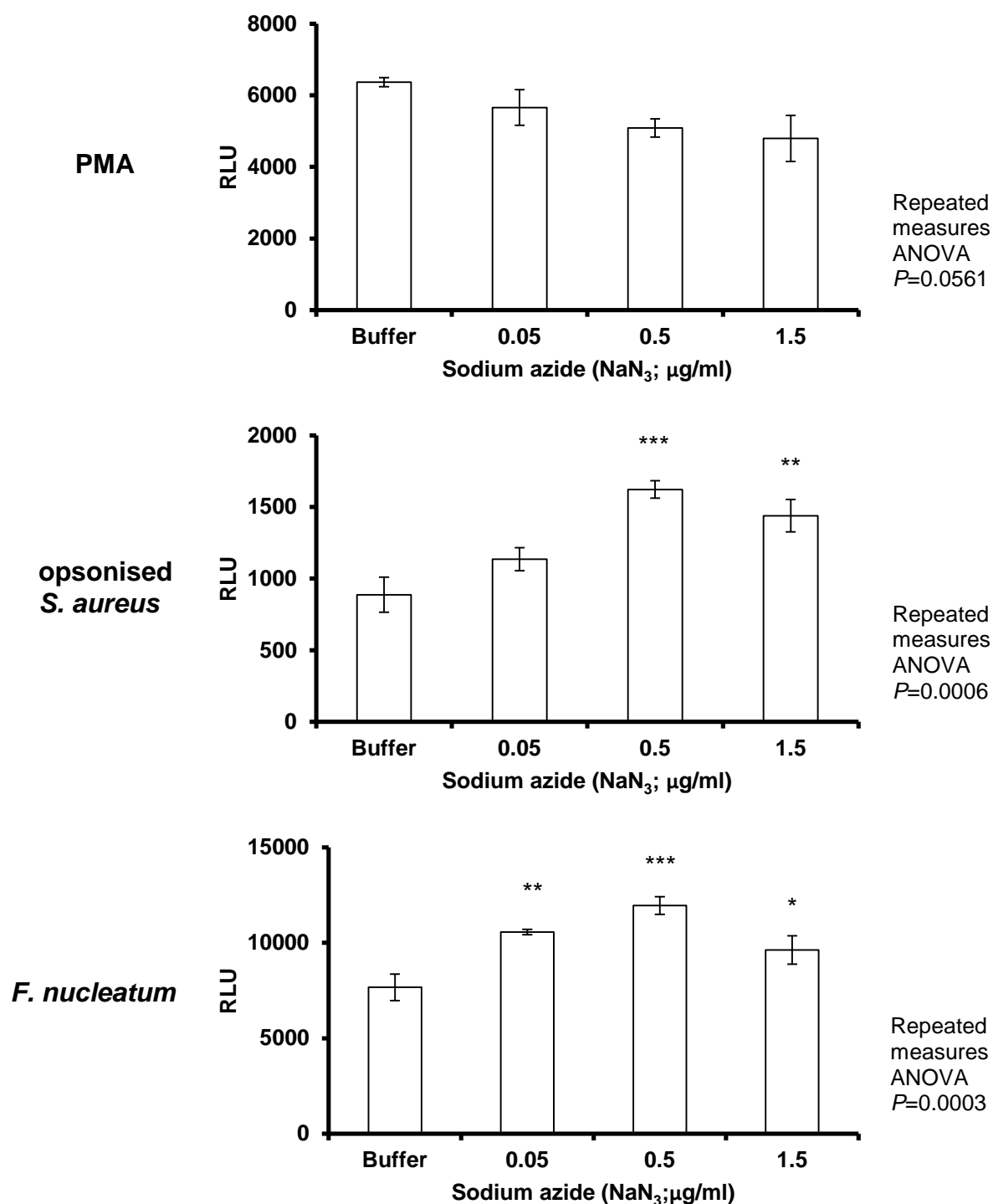


**Figure 7.7. Luminol-detected neutrophil ROS (detected predominantly as intracellular HOCl) generation following 30 minute pre-incubation with sodium azide and subsequent stimulation with PMA (5nM), opsonised *S. aureus* (MOI 300:1) and *F. nucleatum* (MOI 100:1).** Data presented as mean peak relative light units (RLU)  $\pm$  standard deviation and representative of a single experiment performed in triplicate for opsonised *S. aureus* and *F. nucleatum*, and in duplicate for PMA stimulation. *P*-values for comparisons with buffer/0 $\mu\text{g/ml}$  azide-treated cells determined by Bonferroni multiple comparisons test: \*\**P*<0.01, \*\*\**P*<0.001.



**Figure 7.8.** Isoluminol-detected neutrophil ROS (detected predominantly as extracellular HOCl) generation following 30 minute pre-incubation with sodium azide and subsequent stimulation with PMA (5nM), opsonised *S. aureus* (MOI 300:1) and *F. nucleatum* (MOI 100:1). Data presented as mean peak relative light units (RLU)  $\pm$  standard deviation and are representative of a single experiment performed in triplicate for opsonised *S. aureus* and *F. nucleatum*, and in duplicate for PMA stimulation. *P*-values for comparisons with buffer/0µg/ml azide-treated cells determined by Bonferroni multiple comparisons test: \*\**P*<0.01, \*\*\**P*<0.001.





**Figure 7.9.** Lucigenin-detected neutrophil ROS (detected predominantly as extracellular superoxide) generation following 30 minute pre-incubation with sodium azide and subsequent stimulation with PMA (5nM), opsonised *S. aureus* (MOI 300:1) and *F. nucleatum* (MOI 100:1). Data presented as mean peak relative light units (RLU)  $\pm$  standard deviation and are representative of a single experiment performed in triplicate for opsonised *S. aureus* and *F. nucleatum*, and in duplicate for PMA stimulation.  $P$ -values for comparisons with buffer/0 $\mu\text{g/ml}$  azide-treated cells determined by Bonferroni multiple comparisons test: \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

## 7.6 NEUTROPHIL VIABILITY IN THE PRESENCE OF CRP CONTAINING AZIDE

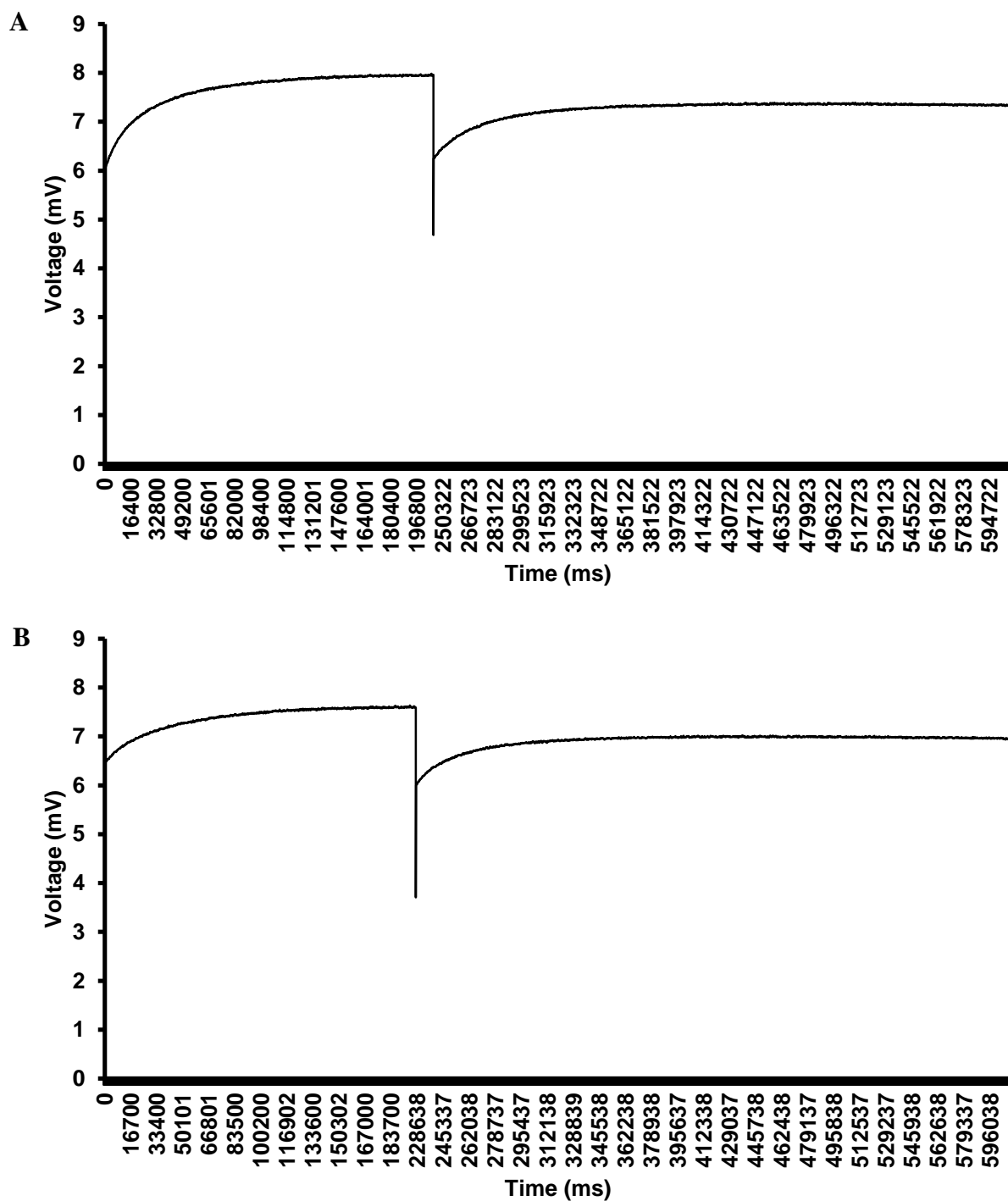
Addition of 1 and 6 µg/ml commercial CRP (i.e. containing 0.5 and 3 µg/ml sodium azide) significantly affected neutrophil viability following a 30 minute incubation ( $P=0.0007$ ; Table 7.2). Neutrophil viability was significantly reduced by 8% and 5% in the presence of 1 µg/ml and 6 µg/ml commercial CRP respectively ( $P<0.01$ ).

RLU	Buffer	CRP (µg/ml)	
		1	6
Mean	283886	262293	268226
SD	14585	14559	6364
<b>Repeated measures ANOVA</b>			
$P=0.0007$			
<b>Bonferroni multiple comparisons test</b>			
$P<0.001$			
$P<0.01$			
ns			

**Table 7.2. Neutrophil viability following 30 minute incubation with 1 and 6 µg/ml CRP determined using Cell-Titre Glo.** Data presented as relative light units (RLU; mean and standard deviation), and representative of 2 experiments performed in triplicate.  $P$ -values calculated using repeated measures ANOVA followed by Bonferroni multiple comparisons test (groups compared in table= ; ns = not significant).

## 7.7 ANTIOXIDANT CAPACITY OF CRP

Whilst in the total antioxidant capacity assay employed the recovery time to T10% is traditionally used as the index for calculating antioxidant capacity (Brock *et al.* 2004; Chapple *et al.* 2007), light output was not inhibited sufficiently to obtain a T10%. Therefore the potential antioxidant capacity of azide-free CRP was calculated as the percentage reduction in light output compared with vehicle control (azide-free Tris buffer; section 2.2.11.1). 0.2µg/ml and 4µg/ml azide-free CRP resulted in a  $0.45 \pm 2.5\%$  and  $0.78 \pm 1.08\%$  reduction in light output respectively (Figure 7.10).



**Figure 7.10.** Typical recovery curves after addition of vehicle control (sodium azide-free Tris buffer; (A) and 4µg/ml dialysed CRP (B) in the chemiluminescence assay to determine antioxidant capacity.

## 7.8 DISCUSSION

The experiments performed to characterise the commercial CRP samples as part of this chapter confirmed the presence of pentameric CRP using analytical ultracentrifugation along with 98% sample purity determined by mass spectrometry. Whilst Tris buffer did not have any direct effect on luminol, isoluminol and lucigenin chemiluminescence, significant increases were detected with regards to H<sub>2</sub>O<sub>2</sub>-induced chemiluminescence using a cell-free system. In addition, equivalent concentrations of sodium azide to those found in the commercial CRP samples had a number of significant effects on unstimulated and FcγR, TLR and PMA stimulated neutrophil ROS generation and release. Furthermore, pre-incubation with commercial CRP (1 & 6µg/ml) containing sodium azide (0.5 and 3µg/ml) caused a small but significant reduction in neutrophil viability.

Human CRP used for *in-vitro* studies may be obtained from a number of different sources including recombinant (Tanaka *et al.* 2002, Pepys *et al.* 2005, Fujii *et al.* 2006), malignant ascetic fluid (Blizniukov *et al.* 2003, Clapp *et al.* 2005, Pepys *et al.* 2005), pleural fluids (Du Clos *et al.* 1991, Zeller & Sullivan 1992, Stein *et al.* 2000) and commercial samples (Khreiss *et al.* 2002, 2005, Verma *et al.* 2002, Hart *et al.* 2005, Biro *et al.* 2007). CRP samples are occasionally documented as being characterised before use in *in-vitro* methodology, however the majority of studies do not publish details of protein characterisation or buffer contents which is important regarding the accuracy of *in-vitro* methodology and also when comparing datasets. Indeed, it has been postulated that different buffer constituents may be responsible for producing artefactual data as a result of CRP buffer constituents rather than due to the CRP itself (Ginsburg *et al.* 1993, Zouki *et al.* 2001, Khreiss *et al.* 2002, 2005, Fujii *et al.* 2006, Freitas *et al.* 2009).

It is therefore disappointing that many authors do not completely reveal the composition and/or concentrations of incubation buffers when publishing *in vitro* methodology and this is not solely limited to studying the effects of CRP. This is a potentially very important issue especially when studying respiratory burst activity since it has been known for several years that components of buffer media alone have the potential to influence the chemiluminescent signals independently of the presence and number of cells included within the assay (Nelson *et al.* 1977). Therefore all subsequent CRP experiments performed within this thesis (Chapter

8) were controlled within the assay itself with the addition of CRP samples alongside dialysed buffer minus CRP as vehicle control, given that Tris buffer alone increases the H<sub>2</sub>O<sub>2</sub>-induced chemiluminescence signal as presented in this chapter. The CRP samples used in this thesis were commercially supplied in the presence of a Tris buffer (Bleich & Schmartz 1966) containing 280mM sodium chloride, 5mM calcium chloride and 0.1% sodium azide as a preservative. Whilst the addition of Tris buffer minus sodium azide did not directly affect the luminol, isoluminol and lucigenin induced chemiluminescent signal using a cell-free system, in agreement with studies investigating the role of buffers on neutrophil chemiluminescence (Ginsburg *et al.* 1993), it did cause a small but noticeable increase in H<sub>2</sub>O<sub>2</sub>-induced chemiluminescent signal. Furthermore, a significant moderate positive correlation between Tris buffer concentration and H<sub>2</sub>O<sub>2</sub>-induced luminol, isoluminol and lucigenin chemiluminescence were demonstrated.

Along with the effects of buffer constituents, there is emerging awareness of the use of commercial sample preparations for *in vitro* studies, with many of these samples both incompletely defined and/or subsequently found to contain additional biologically active agents such as sodium azide which is a commonly used sample preservative. The presence of concurrent sodium azide is of particular interest and importance when investigating neutrophil biology given that it is known to inhibit important neutrophil enzymes including MPO and catalase activity (Ortiz de Montellano *et al.* 1988, Van Uffelen *et al.* 1998). The data presented in this chapter demonstrates that sodium azide inhibits both unstimulated as well as FcγR and TLR stimulated neutrophil ROS generation in agreement with previous studies investigating neutrophil luminol chemiluminescent responses (Bednar *et al.* 1996, Hasegawa *et al.* 1997, Hitzfeld *et al.* 1997, Razumovitch *et al.* 2004). Furthermore, luminol chemiluminescence has been shown to be completely inhibited by 0.3mM sodium azide (Razumovitch *et al.* 2004), and this is likely to be due to reduced conversion of H<sub>2</sub>O<sub>2</sub> to HOCl following inhibition of neutrophil MPO by sodium azide, although there are currently no known studies confirming the ability of sodium azide to traverse cell membranes of viable neutrophils. In contrast to luminol and isoluminol chemiluminescence, the data presented in this chapter also demonstrated greater FcγR and TLR, but with no significant differences in PMA stimulated lucigenin-detected neutrophil O<sub>2</sub><sup>-</sup> release. This is in agreement with previous studies showing that sodium azide enhances lucigenin chemiluminescence, although with a

differential effect following PMA stimulation (Hasegawa *et al.* 1997, Allen 1986). It is possible that the elevated lucigenin-enhanced chemiluminescence reflects increased accumulation of  $O_2^-$  due to blockade of  $O_2^-$  and  $H_2O_2$  consumption by sodium azide inhibition of MPO thereby leading to increased extracellular  $O_2^-$  release (Figure 5.5). The contrasting, albeit not significant, reduction in PMA stimulated  $O_2^-$  release may relate to a previously reported increase in PMA-activated neutrophil apoptosis in the presence of sodium azide (Lundqvist-Gustafsson & Bengtsson 1999).

It is therefore possible that additional sample constituents have biological effects on neutrophil function and interestingly CRP-induced *in vitro* vasorelaxation is now thought to be artefactual and caused by the concurrent presence of sodium azide (van den Berg *et al.* 2004, Liu *et al.* 2005, Swafford *et al.* 2005). It has also been reported that the biological effects of commercial CRP preparations could be completely abrogated by dialysis to remove contaminating sodium azide, and also that the inclusion of azide at equivalent concentrations completely reinstated all of the commercial preparation effects *in vitro* (Liu *et al.* 2005). In addition, many of the proinflammatory, proapoptotic, antiproliferative, antimigratory and antiangiogenic effects attributed to CRP have similarly been associated with the presence of sodium azide and/or contaminating LPS (Schwedler *et al.* 2006, Liu *et al.* 2005, Taylor *et al.* 2005, Yeh 2005). Whilst a number of studies include the removal of sodium azide by dialysis or specifically state buffers to be free of sodium azide (Hart *et al.* 2005, Khreiss *et al.* 2005, Pepys *et al.* 2005, Fujii *et al.* 2006), others, using commercial CRP samples, do not address the problems associated with the presence of sodium azide. The data presented in this chapter is in agreement with Liu and co-workers (2005) who suggest that future studies with commercial CRP should be performed following CRP separation from sodium azide, authentication of biological findings with use of azide concentrations equal to those found in commercial preparation, or alternatively, the use of a recombinant CRP source that should be sterile and not require addition of preservative.

Many studies investigating CRP do not test its effect on cell viability, particularly when using concentrations that are towards the higher end of those that are physiologically relevant. This is important given that any observed changes in neutrophil function in the presence of CRP may otherwise be due to cell necrosis or apoptosis, rather than an alteration in viable cell

physiology. However, the data presented in this thesis demonstrated that  $\leq 6\mu\text{g/ml}$  CRP had a negligible effect on neutrophil viability in agreement with a previous study showing that  $<50\mu\text{g/ml}$  CRP has no effect of neutrophil viability measured by trypan blue dye exclusion (Zhong *et al.* 1998).

The antioxidant effects of physiologically-relevant concentrations of CRP were also investigated and presented in this chapter. Given the published reports investigating the effects of CRP on the respiratory burst, it is interesting that there are currently no known published studies investigating the antioxidant potential of CRP. The CRP antioxidant data presented in this thesis therefore represents novel data that is relevant to data interpretation when investigating the effects of CRP on neutrophil ROS generation presented in Chapter 8. The data confirms that the antioxidant capacity of CRP, as measured using this validated assay is negligible and unlikely to have any significant *in vivo* relevance in itself.

Overall, the data presented in this chapter confirmed the presence of pentameric CRP within the commercial CRP sample, along with determining the effect of CRP on neutrophil viability and antioxidant potential for the first time. Furthermore, the data presented in this Chapter demonstrate the importance of characterising protein content, and concurrent buffer constituents with *in-vitro* methodologies. For example, the effects of Tris buffer on cell-free  $\text{H}_2\text{O}_2$ -induced chemiluminescence demonstrate the importance of incorporating a vehicle control with *in vitro* methodology. In addition, the effects of equivalent concentrations of sodium azide highlighted the need for sample dialysis (section 2.2.11.2) prior to investigating the effects of CRP on the neutrophil respiratory burst presented in Chapter 8.



# **CHAPTER 8**

## **RESULTS & DISCUSSION**

**Effects of C-reactive protein on the neutrophil respiratory burst**

## 8.1 INTRODUCTION

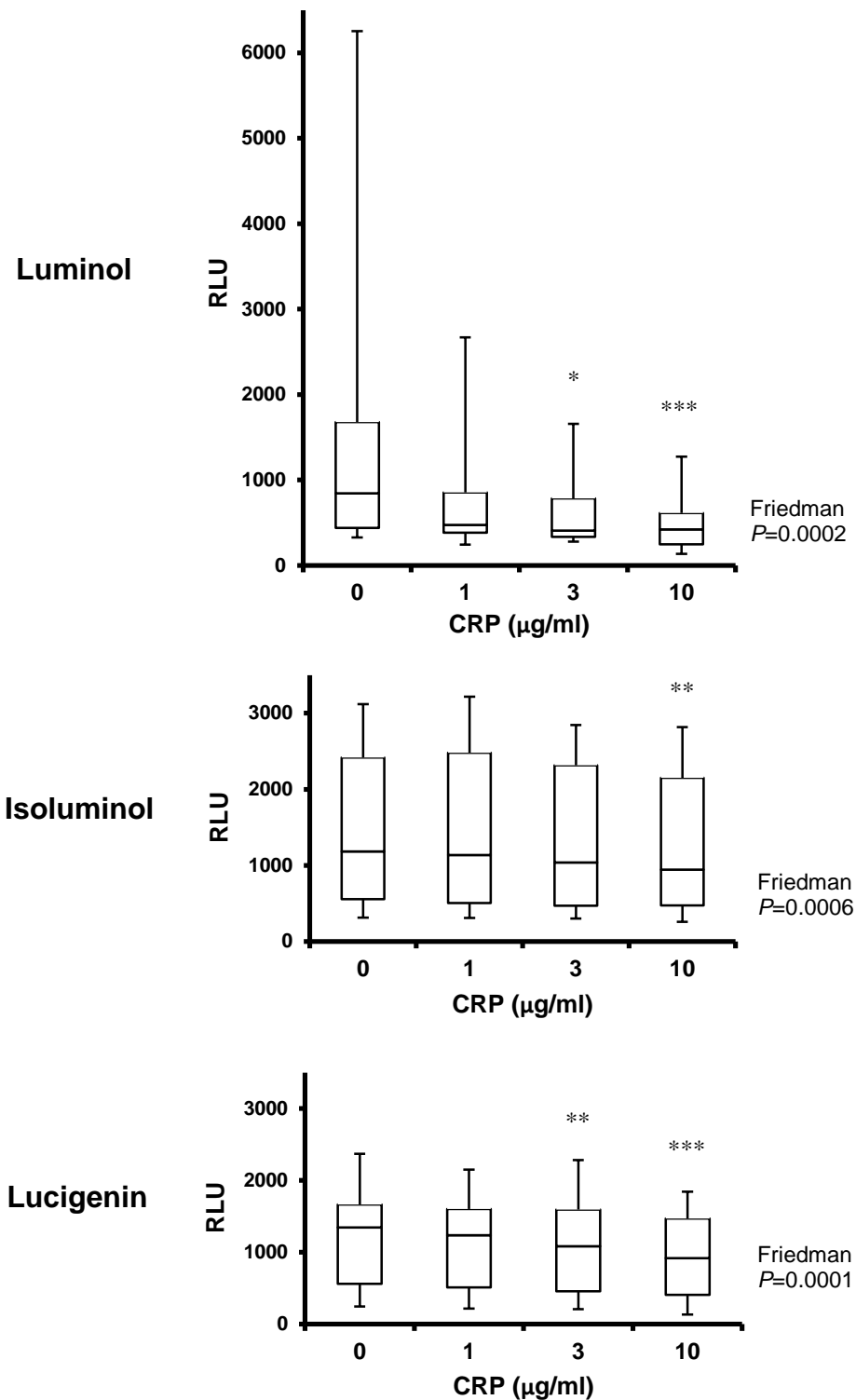
The effects of physiologically-relevant concentrations of CRP on the neutrophil respiratory burst were determined using chemiluminescence methods. Having demonstrated that some components within the buffer in which commercial CRP is supplied alter chemiluminescence assays, CRP samples were extensively dialysed to remove sodium azide and filter-sterilised (section 2.2.11.2). Furthermore, all CRP assays included vehicle controls containing identical amounts of dialysed Tris buffer. The C-reactive protein concentrations tested were chosen to include the systemic concentrations identified from the patient volunteers diagnosed with chronic periodontitis recruited as part of the longitudinal interventional case controlled study (section 3.2) and those associated with cardiovascular disease risk (Ridker *et al.* 2005). The aim was to contribute to our current understanding of neutrophil function in relation to systemic inflammatory markers that are associated with both chronic periodontitis and systemic disease.

## 8.2 EFFECTS OF CRP ON NEUTROPHIL ROS GENERATION

Unstimulated and stimulated neutrophil ROS generation was determined in the presence and absence of pentameric CRP (1, 3 and 10 $\mu$ g/ml) using luminol, isoluminol and lucigenin-enhanced chemiluminescence (section 2.2.11.8).

### 8.2.1 Unstimulated ROS generation in the presence of CRP

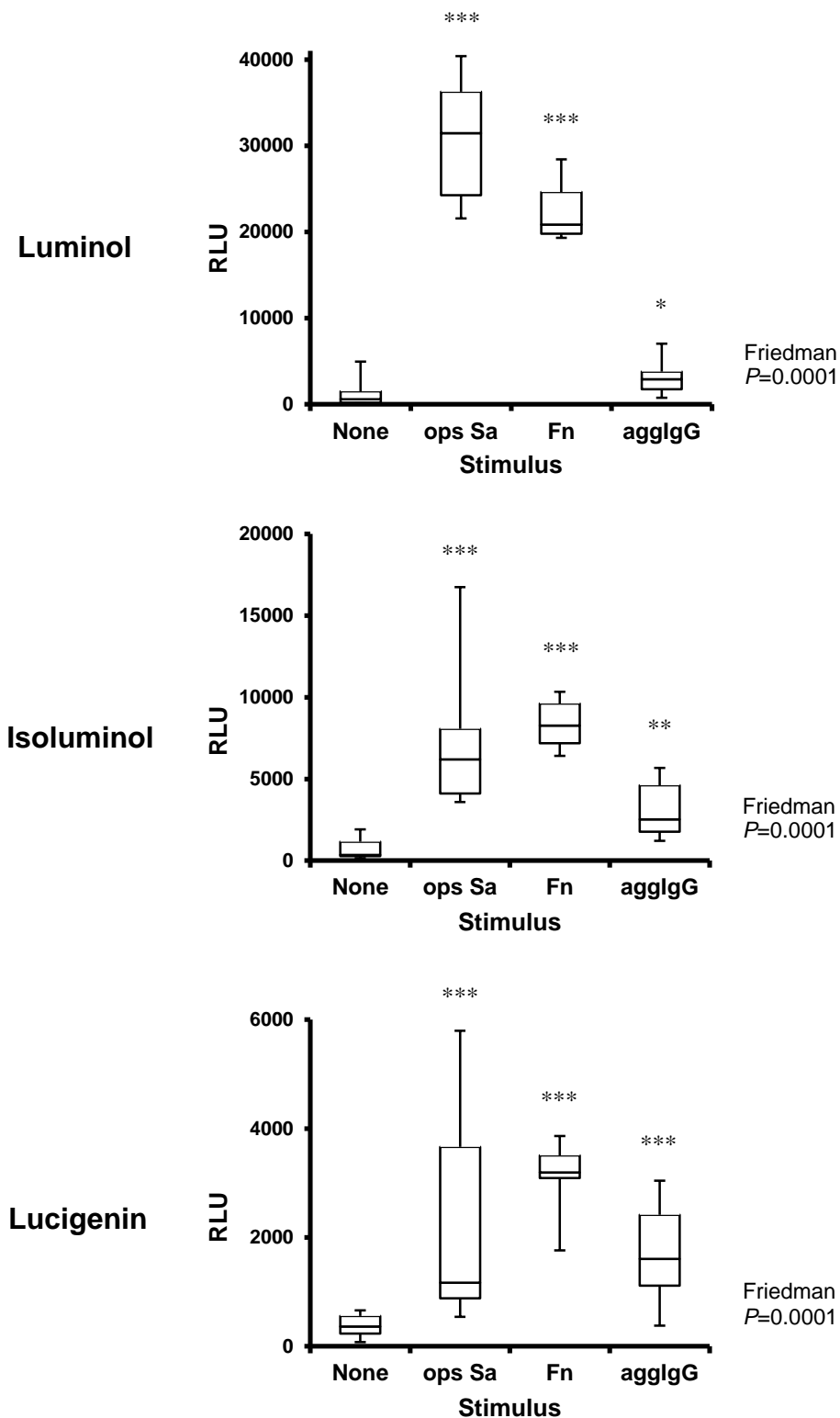
Addition of 1, 3 and 10 $\mu$ g/ml CRP significantly altered neutrophil ROS generation detected by all 3 chemiluminescent substrates in a dose-dependent manner compared to vehicle control (20mM Tris buffer;  $P \leq 0.0006$ ; Figure 8.1). 10 $\mu$ g/ml CRP significantly reduced neutrophil ROS generation detected by luminol, isoluminol and lucigenin-enhanced chemiluminescence compared to vehicle control ( $P \leq 0.01$ ). The mean percentage reductions in unstimulated ROS with 10 $\mu$ g/ml CRP were 41.8%, 11.9% and 28.8% for luminol, isoluminol and lucigenin respectively. In addition, 3 $\mu$ g/ml CRP also significantly reduced neutrophil intracellular HOCl and extracellular O<sub>2</sub><sup>-</sup> release detected by luminol and lucigenin respectively ( $P \leq 0.05$ ; Figures 8.1).



**Figure 8.1.** The effect of CRP on unstimulated ROS generation detected by luminol, isoluminol and lucigenin. Peak chemiluminescence (median, interquartile range, maximum and minimum RLU;  $n=14$ ) attained during the 30 minutes after addition of CRP (1, 3 & 10  $\mu\text{g/ml}$ ) or vehicle control (0  $\mu\text{g/ml}$  CRP). Significant differences from vehicle control-treated cells determined using Dunn's multiple comparisons test: \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

### **8.2.2 Confirmation of increased neutrophil ROS generation after neutrophil FcγR and TLR-stimulation.**

Neutrophils were stimulated after an initial 30 minute baseline resting period and an additional 30 minutes following addition of dialysed Tris buffer (CRP vehicle control). Addition of IgG-opsonised *S. aureus* (MOI 300:1), *F. nucleatum* (MOI 100:1) and aggregated IgG (600µg/ml final well concentration) significantly increased neutrophil ROS generation detected by all 3 chemiluminescent substrates ( $P=0.0001$ ; Figure 8.2). Stimulation of luminol detectable ROS generation was significantly higher following addition of opsonised *S. aureus* compared to aggregated IgG ( $P<0.05$ ) and, whereas median levels of isoluminol and lucigenin detectable ROS release were also greater after stimulation with opsonised *S. aureus*, the difference between these two FcγR stimuli were not statistically significant.

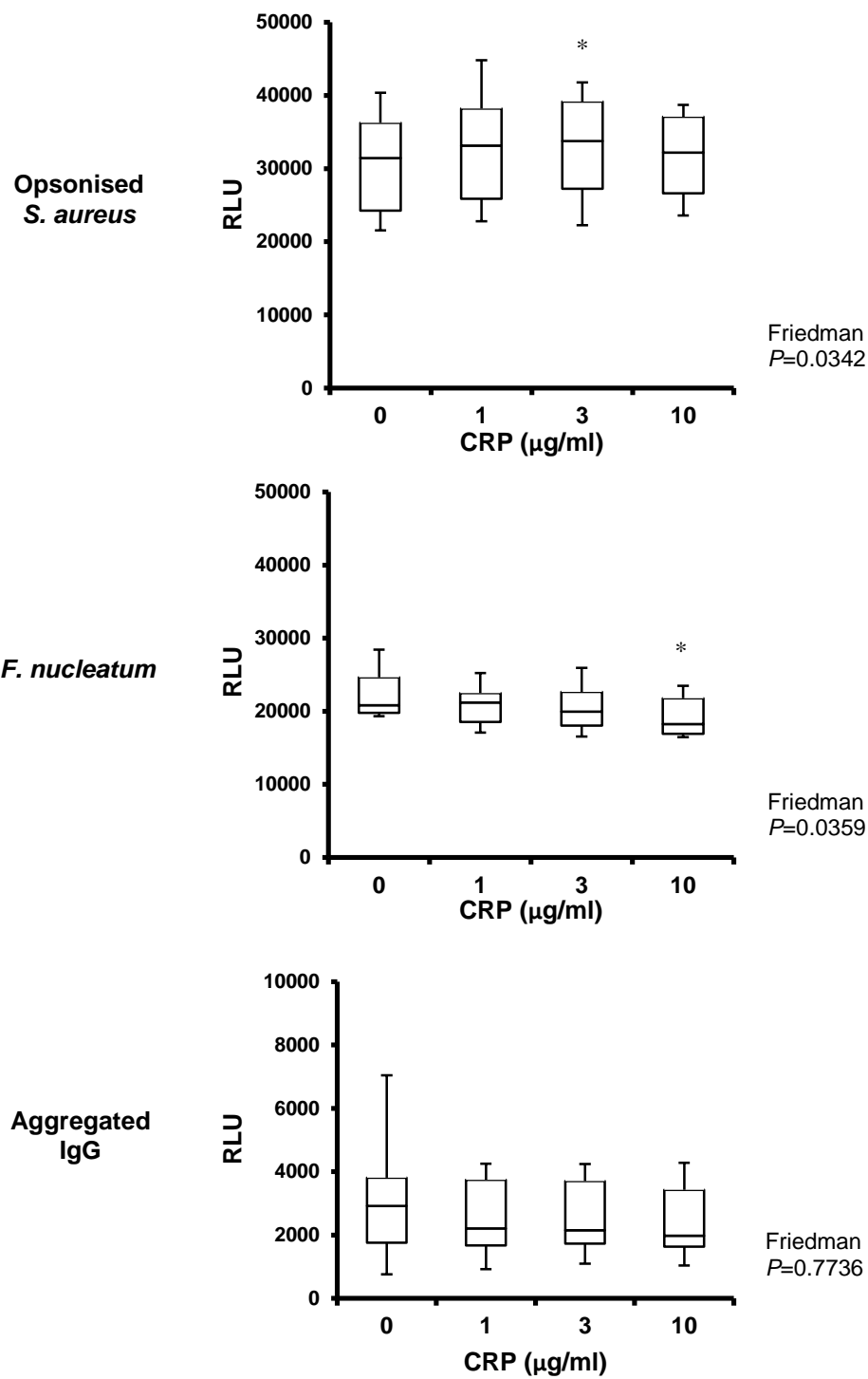


**Figure 8.2. Neutrophil ROS generation and release after a 30 minute pre-incubation with Tris buffer (CRP vehicle control) and subsequent stimulation with opsonised *S. aureus* (ops Sa; MOI 300:1), *F. nucleatum* (Fn; MOI 100:1) and aggregated IgG (agglgG; 600µg/ml final well concentration) detected by luminol, isoluminol and lucigenin. Significant differences from baseline chemiluminescence determined using Dunn's multiple comparisons test: \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .**

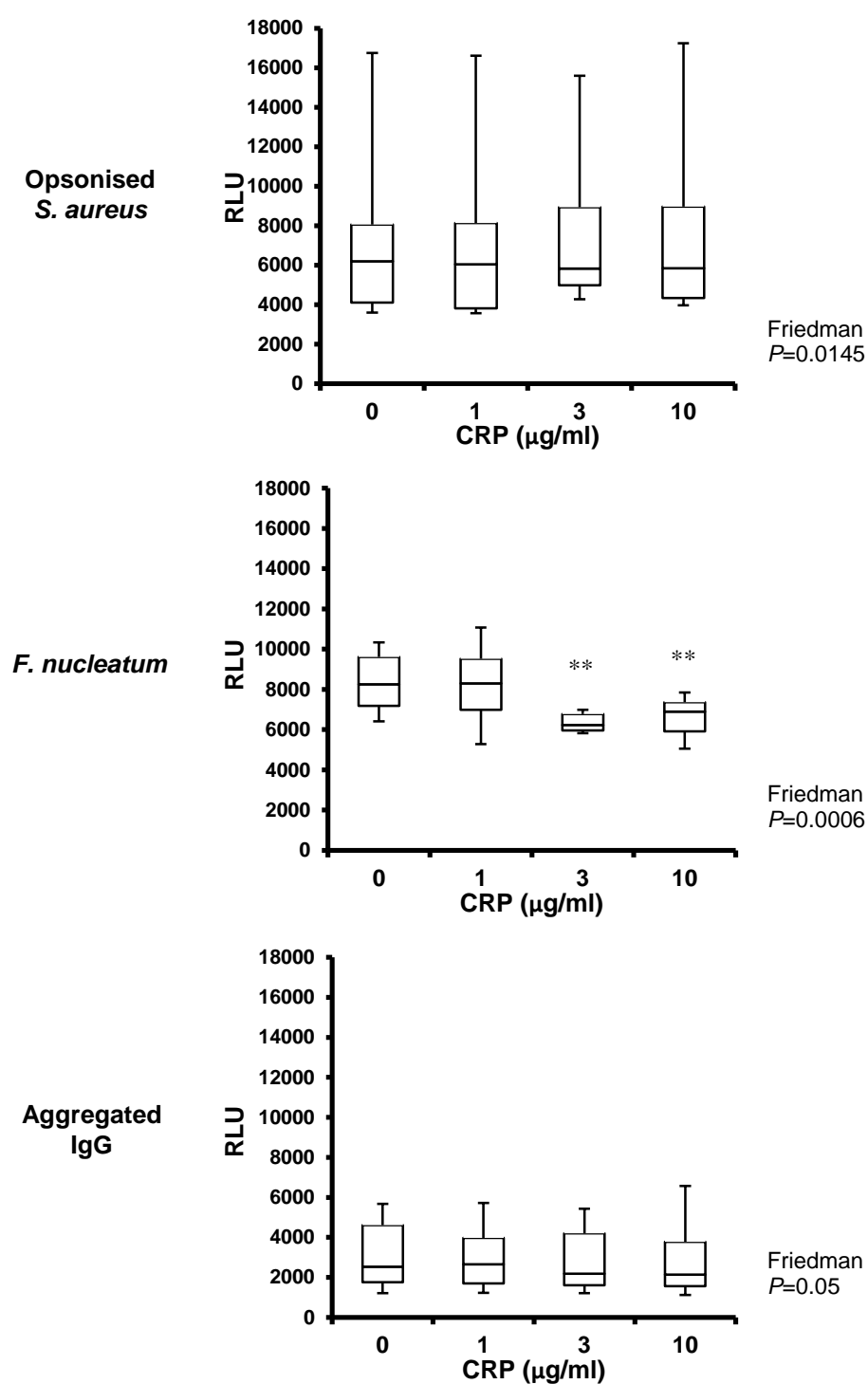
### 8.2.3 Stimulated ROS generation in the presence of CRP

Neutrophil stimulation was performed following a 30 minute baseline resting period and a 30 minute pre-incubation with CRP. There was no clear relationship between FcγR-stimulated neutrophil ROS generation in the presence of CRP detected by luminol and isoluminol chemiluminescence (Figures 8.3 & 8.4). Whilst median levels of luminol-detected intracellular HOCl following stimulation with IgG-opsonised *S. aureus* were higher in the presence of CRP, this was only significant for 3μg/ml CRP, and was not present following stimulation with aggregated IgG. In contrast, a small dose-dependent decrease in neutrophil O<sub>2</sub><sup>-</sup> release detected by lucigenin was observed, which was significant in the presence of 10μg/ml CRP following FcγR-stimulation with both IgG-opsonised *S. aureus* (13.9% reduction; *P*<0.001) and aggregated IgG (17.9% reduction; *P*<0.05).

*F. nucleatum* TLR-stimulated neutrophil ROS generation was significantly altered in the presence of CRP as detected by all 3 chemiluminescent substrates (*P*<0.0359; Figures 8.3, 8.4 & 8.5). There was a small dose-dependent decrease in TLR-stimulated neutrophil intracellular HOCl detected by luminol that was significant in the presence of 10μg/ml CRP (13.4% reduction; *P*<0.05). However, the predominant effect of CRP related to TLR-stimulated extracellular ROS detected by isoluminol and lucigenin (Figures 8.4 & 8.5). There was a significant reduction in TLR-stimulated extracellular ROS detected by isoluminol in the presence of 3 and 10μg/ml CRP reducing H<sub>2</sub>O<sub>2</sub>/HOCl release by 22.5% and 19.2% respectively (*P*<0.01; Figure 8.4). In contrast, CRP increased TLR-stimulated neutrophil O<sub>2</sub><sup>-</sup> release detected by lucigenin at all concentrations tested, with a mean increase of 21.5% (*P*<0.05 for 1 and 3μg/ml CRP; *P*<0.001 for 10μg/ml CRP; Figure 8.5).

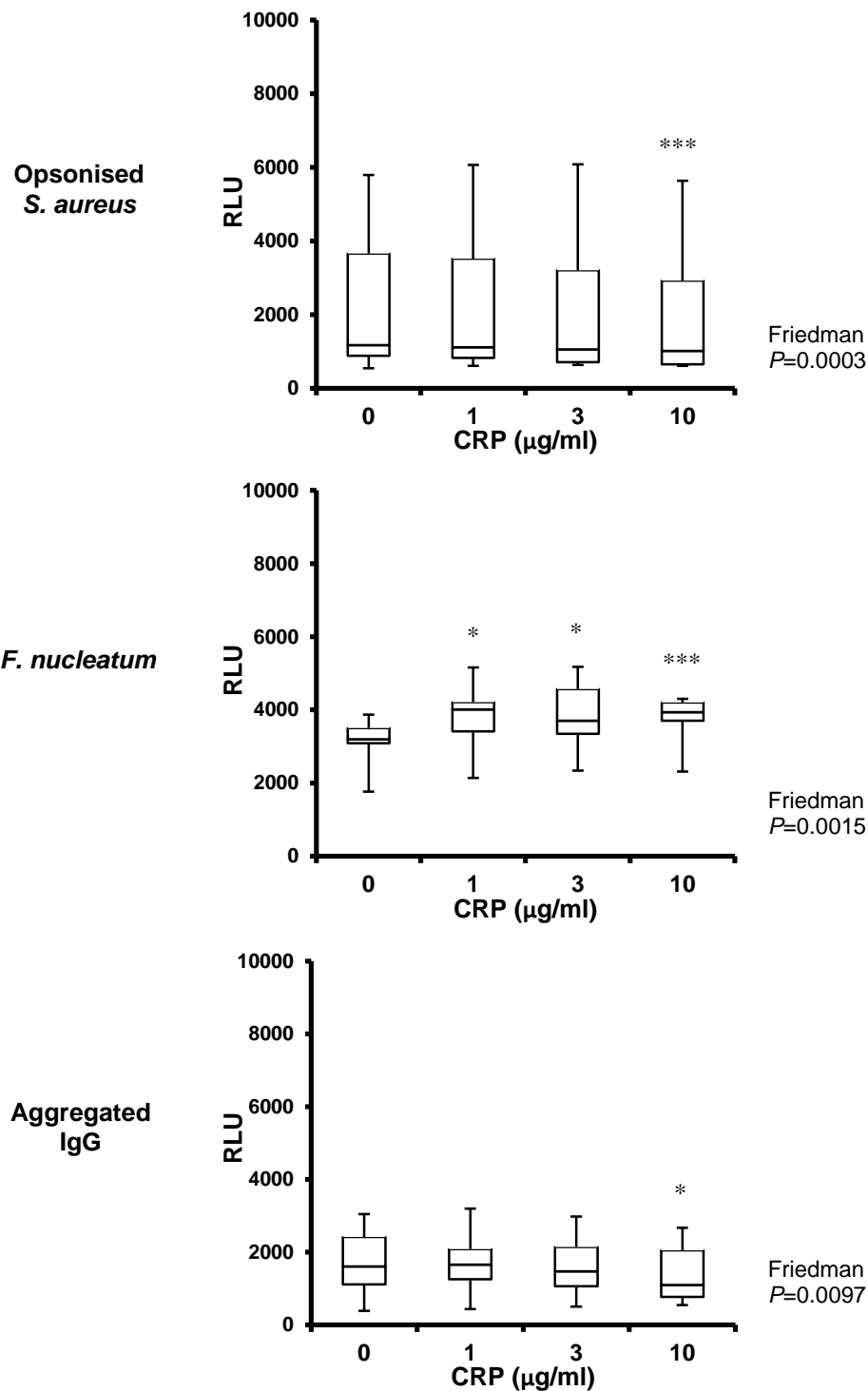


**Figure 8.3.** Luminol-detected neutrophil ROS (detected predominantly as intracellular HOCl) generation following 30 minute pre-incubation with CRP and subsequent stimulation with IgG-opsonised *S. aureus* (MOI 300:1), *F. nucleatum* (MOI 100:1) and aggregated IgG (600µg/ml final well concentration). Peak chemiluminescence (median, interquartile range, maximum and minimum RLU; n=14) attained after stimulation. Significant differences from vehicle control-treated cells determined using Dunn's multiple comparisons test: \* $P<0.05$ .



**Figure 8.4.** Isoluminol-detected neutrophil ROS (detected predominantly as extracellular HOCl) generation following 30 minute pre-incubation with CRP and subsequent stimulation with IgG-opsonised *S. aureus* (MOI 300:1), *F. nucleatum* (MOI 100:1) and aggregated IgG (600µg/ml final well concentration). Peak chemiluminescence (median, interquartile range, maximum and minimum RLU; n=14) attained after stimulation. Significant differences from vehicle control-treated cells determined using Dunn's multiple comparisons test: \*\* $P<0.01$ .





**Figure 8.5.** Lucigenin-detected neutrophil ROS (detected as extracellular superoxide) generation following 30 minute pre-incubation with CRP and subsequent stimulation with IgG-opsonised *S. aureus* (MOI 300:1), *F. nucleatum* (MOI 100:1) and aggregated IgG (600µg/ml final well concentration). Peak chemiluminescence (median, interquartile range, maximum and minimum RLU; n=14) attained after stimulation. Significant differences from vehicle control-treated cells determined using Dunn's multiple comparisons test: \* $P<0.05$ , \*\*\* $P<0.001$ .

### 8.3 DISCUSSION

The data presented within this chapter demonstrates, for the first time, that physiologically-relevant ( $\leq 10\mu\text{g/ml}$ ) concentrations of pentameric CRP have the ability to reduce unstimulated neutrophil ROS generation. Furthermore, CRP reduced Fc $\gamma$ R stimulated neutrophil O $_2^-$  release and, whilst decreasing TLR stimulated HOCl generation, increased TLR stimulated extracellular O $_2^-$  release.

Systemic CRP concentrations are raised in a number of inflammatory conditions, including cardiovascular disease (Ridker *et al.* 2005) and chronic periodontitis (Paraskevas *et al.* 2008), and are widely used medically as a gauge of systemic inflammation (Ballou & Kushner 1992). Serum CRP concentrations may increase to  $>500\text{mg/l}$  within 24-74 hours as part of the systemic inflammatory response to infection or tissue damage (Pepys & Hirschfield 2003). Serum CRP levels are also of prognostic value in predicting acute cardiovascular events (Ridker *et al.* 1997, 2005). Greater plasma CRP concentrations were identified in patients compared to the healthy control volunteers recruited for the longitudinal intervention case-control clinical study presented in this thesis. The median plasma CRP concentration in patients was  $2.15\text{mg/l}$  CRP (section 3.4) and in agreement with  $1\text{-}4\text{mg/l}$  CRP previously reported in chronic periodontitis (Paraskevas *et al.* 2008). Thus, equivalent CRP concentrations were therefore utilised in this chapter to determine its effect upon neutrophil ROS generation.

There is evidence that CRP concentrations progressively increase in periodontal health through to chronic gingivitis and chronic periodontitis. Serum, salivary and GCF CRP concentrations have all been shown to be higher in patients with chronic gingivitis compared to healthy controls, albeit not significantly (Herrera *et al.* 2007, Alzahrani *et al.* 2013, Patil & Desai 2013, Shojaee *et al.* 2013) possibly relating to the lack of systemic involvement in chronic gingivitis. Similarly, there were no significant differences in serum CRP concentrations detected during a 21-day model of experimental gingivitis (Wahaidi *et al.* 2011), possibly relating to acute-onset rather than chronic inflammation associated with clinically-diagnosed chronic gingivitis.

Increased neutrophil ROS release, collateral host tissue damage and the formation of oxidative stress are established pathological features of chronic periodontitis and are associated with systemic disease (Chapple & Matthews 2007). C-reactive protein has the potential to modify a number of different neutrophil functions since CRP accumulates at sites of inflammation associated with infiltrating neutrophils (Shephard *et al.* 1990). Furthermore, CRP concentrations are raised in conditions associated with oxidative stress, including cardiovascular disease (Ridker *et al.* 2005) and chronic periodontitis (Paraskevas *et al.* 2008). The data presented in this chapter demonstrates, for the first time, that CRP, at concentrations equivalent to those found in the plasma of chronic periodontitis patients, significantly reduce unstimulated neutrophil ROS release, with a 12-42% reduction in unstimulated neutrophil ROS detected with all 3 chemiluminescent substrates. This suggests a protective role for CRP in reducing the excess hyperactive neutrophil ROS generation and release observed in patients with chronic periodontitis (Matthews *et al.* 2007; Chapter 5), reducing the risk of collateral tissue damage and also co-morbidity associated with the presence of oxidative stress.

The negligible antioxidant properties of CRP, as measured by luminol chemiluminescence (<1% reduction in chemiluminescence signal; section 7.7), cannot account for the reduction in unstimulated neutrophil ROS generation presented in this chapter. Furthermore, reduced luminol-detected intracellular HOCl generation cannot be due to simple protein scavenging since CRP is unable to enter the cell (Bottazzi *et al.* 2010). Therefore, it is possible that the reduction in unstimulated neutrophil ROS generation results from a reduction in NADPH oxidase activation in the cell membrane and intracellular granule membrane resulting from a CRP-FcγR interaction (Heuertz *et al.* 2005).

The presence of CRP also had a number of significant effects on stimulated neutrophil ROS generation, which varied according to the stimuli and nature of the ROS measured. This is the first study to measure FcγR-stimulated neutrophil ROS release using physiologically-relevant stimuli in the presence of physiologically-relevant concentrations of CRP. C-reactive protein had no consistent effects on FcγR stimulated intra- or extracellular HOCl generation but significantly reduced O<sub>2</sub><sup>-</sup> release. That the data for isoluminol-detectable extracellular HOCl showed no significant differences in the presence of CRP may reflect the fact that extracellular HOCl is derived from both membrane-released O<sub>2</sub><sup>-</sup> and diffusion of H<sub>2</sub>O<sub>2</sub> from

within the cell, with the latter masking the small changes in the former seen with lucigenin. The reduction in FcγR-stimulated O<sub>2</sub><sup>-</sup> release caused by low levels of CRP is consistent with current literature demonstrating that high concentrations (>50mg/l) inhibit PMA-, conA and fMLP-stimulated neutrophil O<sub>2</sub><sup>-</sup> release (Buchta *et al.* 1987, Shephard *et al.* 1990, Dobrinich & Spagnuolo 1991, Ratnam & Mookerjea 1998, Mortensen & Zhong 2000). The reduction in FcγR-stimulated O<sub>2</sub><sup>-</sup> presented in this chapter may be the result of receptor blocking/competition since CRP is known to bind to FcγRs (Heuertz *et al.* 2005, Khreiss *et al.* 2005). However this does not completely explain the data given the reported reduction in PMA, conA and fMLP stimulated O<sub>2</sub><sup>-</sup> release, which do not activate neutrophils by FcγRs, along with a lack of consistent effect on FcγR-stimulated intra- and extracellular HOCl generation. It is possible that this attenuation of neutrophil ROS generation is a means to minimize tissue damage *in vivo* at sites where CRP accumulates. Furthermore, that the reduced O<sub>2</sub><sup>-</sup> release was not reflected in reduced intracellular luminol-detected HOCl levels suggests retention of neutrophil defence functions requiring intracellular ROS such as phagocytosis (Winterbourn & Kettle 2013) and NET production (Palmer *et al.* 2012).

The ability of physiologically relevant concentrations of CRP to enhance *F. nucleatum* TLR stimulated neutrophil extracellular O<sub>2</sub><sup>-</sup> release, despite a reduction in intra- and extracellular HOCl, is a novel finding reported in this chapter and may represent an important contributing factor in the pathogenesis of chronic periodontitis. The consistent increase in neutrophil extracellular O<sub>2</sub><sup>-</sup> release, despite a reduction in intra- and extracellular HOCl generation following *F. nucleatum* TLR stimulation, could be due to activation and relocation of NADPH oxidase to the neutrophil cell surface. This would result in an increase in extracellular O<sub>2</sub><sup>-</sup> release (detected by lucigenin), with a concurrent decrease in intracellular O<sub>2</sub><sup>-</sup> and generation/release of H<sub>2</sub>O<sub>2</sub> detected predominantly as HOCl by luminol and isoluminol respectively. Furthermore, it is unlikely that CRP would affect SOD and MPO enzyme activities given that they're predominantly intracellular and CRP is unable to enter the cell, with 50μg/ml CRP having been shown to have no significant effect on neutrophil membrane expression of MPO (Xu *et al.* 2012). Interestingly NADPH oxidase relocation to the outer cell membrane has been demonstrated in peripheral blood neutrophils from patients with chronic periodontitis (Dias *et al.* 2013) of which *F. nucleatum* is a widely recognised pathogen.

The increased *F. nucleatum*-stimulated  $O_2^-$  release was consistent, even at the lowest (1 $\mu$ g/ml) CRP concentration tested, and may be the result of Fc $\gamma$ R/TLR cross-linking in the presence of both CRP and *F. nucleatum*. C-reactive protein binds predominantly to phosphorylcholine in the presence of  $Ca^{2+}$ , which is present on a number of different host and bacterial cells, including *F. nucleatum* (Gmur *et al.* 1999, Schenkein *et al.* 2001). It is therefore likely that during stimulation with *F. nucleatum* in the presence of CRP, neutrophil receptor cross-linking could occur with TLR binding *F. nucleatum* and adjacent Fc $\gamma$ R binding with CRP thereby potentially up-regulating the downstream activation events leading to increased translocation of NADPH oxidase to the cell surface and observed increase in  $O_2^-$  release. Furthermore, TLR-Fc $\gamma$ R interactions have been shown to be important in the activation of neutrophils, macrophages and dendritic cells (Rittirsch *et al.* 2009, den Dunnen *et al.* 2012).

Pentameric CRP has also been shown to dissociate and aggregate under certain conditions including heat, acid and urea dissociation and chelation (Potempa *et al.* 1987) thereby forming modified CRP (mCRP) which is insoluble and antigenically distinct. Furthermore, mCRP has been recently shown to be generated on cell membranes of activated platelets associated with vascular injury (Ji *et al.* 2007, Eisenhardt *et al.* 2009) with its occurrence being considered responsible for the pathological features associated with atherosclerosis (Diehl *et al.* 2000, Khreiss *et al.* 2004). All procedures undertaken as part of this thesis were therefore undertaken in  $Ca^{2+}$ -containing buffers to prevent spontaneous dissociation of pentameric CRP and generation of mCRP. Furthermore, the native pentameric structure of CRP was confirmed (section 7.2) prior to investigating the effects of CRP on the neutrophil respiratory burst. Pentameric and mCRP have different biological properties and differentially bind to human neutrophils, with pentameric CRP binding to Fc $\gamma$ RI (CD64) and Fc $\gamma$ RIIa (CD32) and mCRP binding to Fc $\gamma$ RIII (CD16) respectively (Khreiss *et al.* 2005). With regards to the effects of mCRP on the neutrophil respiratory burst, evidence from a single research group indicates that mCRP has no direct effect on baseline neutrophil ROS generation. However, at high concentrations (>50 $\mu$ g/ml), mCRP appeared to increase ROS generation in response to aggregated IgG but not PMA or opsonised zymosan (Zeller *et al.* 1986, 1992).

Overall, the data obtained show that physiologically-relevant concentrations of CRP decrease unstimulated and Fc $\gamma$ R-stimulated O $_2^-$  release by neutrophils potentially offering some degree of host protection by minimising the effects of oxidative stress, systemic inflammation and associated risk of co-morbidity. By contrast, CRP enhanced TLR stimulated neutrophil O $_2^-$  release that has the potential to increase both oxidative stress and the risk of collateral host tissue damage in chronic periodontitis, as well as host protection during the transition from gingivitis to periodontitis when acquired immunity had not been developed.

## **CHAPTER 9**

### **CONCLUDING REMARKS**

## 9.1 SUMMARY OF MAIN FINDINGS

The overall aim of this thesis was to contribute to our current understanding of the role of the neutrophil in the pathogenesis of chronic periodontitis, along with possible associations with systemic disease in which raised systemic CRP concentrations are present.

In order to study novel aspects of neutrophil function in chronic periodontitis patients, a longitudinal intervention case-control clinical study was undertaken (section 2.1.1). Peripheral blood neutrophil cytokine (section 2.2.7) and superoxide (section 2.2.8) release were measured both pre- and post-therapy in patients and matched healthy controls. In addition, NET release (section 2.2.9) and directional chemotactic accuracy (section 2.2.10) were also determined using recently developed assays. An overview of the neutrophil functions studied in relation to the pathogenesis of chronic periodontitis is shown in Figure 9.1.

The neutrophil cytokine release data (Chapter 4) have reaffirmed that neutrophils do not solely function antimicrobially but are also actively involved in co-ordinating the inflammatory-immune response with the hierarchical release of IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  respectively. Furthermore the data demonstrated, for the first time, that peripheral blood neutrophils from patients with chronic periodontitis release greater amounts of IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$ , compared with healthy controls both pre- and post-therapy. Specifically, patient neutrophils were hyper-reactive regarding Fc $\gamma$ R-stimulated IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  and *P. gingivalis* (TLR)-stimulated IL-1 $\beta$  release both before and after periodontal therapy. In contrast, the hyper-reactive, *F. nucleatum* (TLR)-stimulated IL-6, IL-8 and TNF- $\alpha$  release displayed by patient neutrophils was not present at post-periodontal treatment review. Dysregulated neutrophil cytokine release is of significant *in vivo* relevance given that cytokines regulate many different aspects of the inflammatory-immune response, including neutrophil ROS and NET release as well as chemotactic potential (Figure 9.1).





Neutrophil hyper-activity and –reactivity regarding ROS generation is a well-established feature in the pathogenesis of chronic periodontitis (section 1.6.3). Data presented in this thesis have demonstrated patient neutrophil  $O_2^-$  hyperactivity along with *P. gingivalis* and *F. nucleatum* (TLR)-stimulated hyper-reactivity, with no significant periodontitis-associated difference in PMA stimulated extracellular  $O_2^-$  release (Chapter 5). Furthermore, patient neutrophil  $O_2^-$  hyper-activity and *P. gingivalis* and *F. nucleatum* (TLR)-stimulated hyper-reactivity were not present at review, post-treatment. The finding that neutrophil  $O_2^-$  hyper-activity and –reactivity, along with *F. nucleatum* (TLR)-stimulated IL-6, IL-8 and TNF- $\alpha$  hyper-reactivity, were not present at review may possibly be suggestive of acquired rather than constitutional defects in neutrophil function. Acquired defects are likely to result from the permeable nature of the junctional epithelium with periodontal bacteria and their products having the potential to enter the surrounding tissues and vasculature (Loos 2005, Seymour *et al.* 2007) resulting in a systemic inflammatory-immune response. Therefore, the data suggest that removal of supra- and subgingival plaque deposits results in a reduction in peripheral blood neutrophil priming/activation by bacterial plasma-borne components and a consequent correction of the observed neutrophil hyperactivity and –reactivity.

A dysregulated inflammatory-immune response is considered responsible for the majority of disease pathogenesis associated with chronic periodontitis (Page *et al.* 1997; section 1.2.2) however the exact mechanisms remain to be fully elucidated. Dysregulated neutrophil function has the potential to cause collateral host tissue damage as well as delayed bacterial clearance from the gingival crevice resulting in potentiation of a chronic inflammatory lesion. The finding that hyper-reactive, *F. nucleatum* (TLR)-stimulated IL-6, IL-8 and TNF- $\alpha$  and  $O_2^-$  release was absent at review, whereas Fc $\gamma$ R-stimulated IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  and *P. gingivalis* (TLR)-stimulated IL-1 $\beta$  hyper-reactivity persisted post-therapy, is suggestive that both constitutional and acquired defects may underlie the dysregulated peripheral blood neutrophil phenotype in chronic periodontitis. Constitutional defects may involve any aspect of neutrophil function, including Fc $\gamma$ R and *P. gingivalis* TLR stimulated cytokine generation and release. These may represent significant genetic risk factors for chronic periodontitis (Michalowicz 1994). For example several constitutional defects in intracellular signal transduction responsible for chemotaxis and  $O_2^-$  generation have been identified in the pathogenesis of localised aggressive periodontitis (Gronert *et al.* 2004). As a result,

constitutional defects would remain inherent to neutrophil function irrespective of intervention in terms of periodontal treatment.

In addition to acquired and constitutional defects in neutrophil function, recent evidence has also demonstrated that extravasated neutrophils may re-enter the vasculature in a process called “reverse migration” (Mathias *et al.* 2006, Elks *et al.* 2011, Woodfin *et al.* 2011). It is therefore possible that neutrophils recruited to sites of periodontal inflammation may become primed or activated by periodontal bacteria and their products within the inflamed gingival tissues, and then re-enter the circulation resulting in a hyperactive and –reactive peripheral blood neutrophil phenotype. Neutrophil transmigration, contributing 1-2% of the peripheral blood population, has been identified in rheumatoid arthritis (Buckley *et al.* 2006) but has yet to be investigated in chronic periodontitis. It is possible that neutrophil transmigration will have *in vivo* relevance in the pathogenesis of chronic periodontitis given the large surface area of exposed connective tissues and thus vasculature, and the chronic, often long-term nature of disease. However, this is unlikely to be solely responsible for the differences in ROS and cytokine release, presented in this thesis, given the probability that transmigrating cells form a minor population of peripheral blood neutrophils. Whilst neutrophil reverse migration may be beneficial to the host in preserving cells when needed to protect against infection, activated neutrophils re-entering the circulation could disseminate the inflammatory response throughout the body leading to systemic and/or chronic inflammation, and therefore provide a causal link between chronic periodontitis and systemic disease.

Neutrophil extracellular trap (NET) formation is a recently discovered neutrophil antimicrobial mechanism (Brinkmann *et al.* 2004) and considered important in the pathogenesis of chronic periodontitis (reviewed by Cooper *et al.* 2013). Neutrophil extracellular trap release is triggered by ROS, specifically HOCl (Palmer *et al.* 2012). Therefore, given the well-established hyperactive and hyper-reactive ROS generation by neutrophils in periodontitis (section 1.6.3, Chapter 5) NET release was measured, for the first time, using a recently developed fluorometric quantification assay (section 2.2.9). Whilst NET release was confirmed following stimulation with established NET agonists (PMA & HOCl), there were no significant differences in NET release between patient and control cells (Chapter 6). This suggests that the ability to release NETs is unaffected in periodontitis

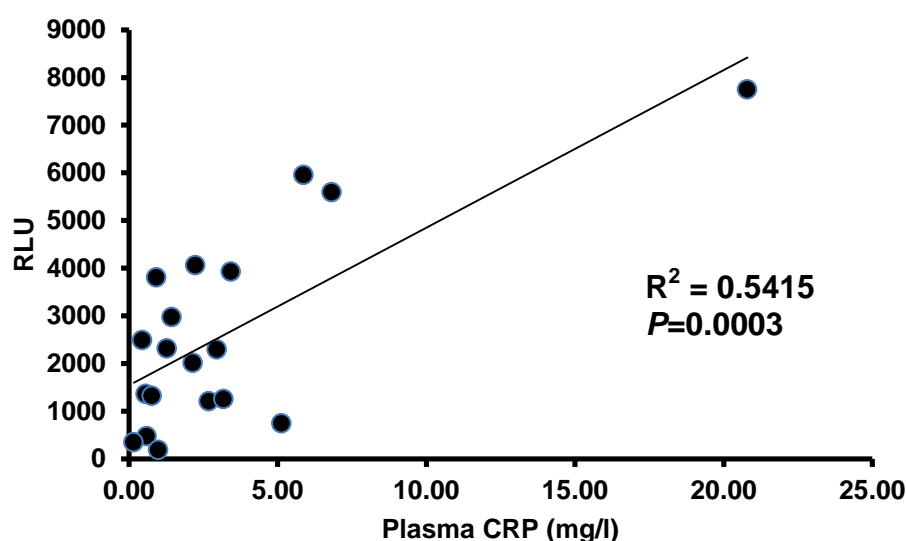
following direct stimulation, however it remains to be elucidated how this may translate to NET release in response to periodontally-relevant bacteria, and within periodontal tissues.

Impaired neutrophil chemotaxis has previously been reported in the pathogenesis of chronic periodontitis (section 1.6.2). This was confirmed and more accurately characterised by the data reported here, generated using a novel directional chemotaxis assay recently developed in collaboration with colleagues at the University of Glasgow (section 2.2.10.1). Patient neutrophils demonstrated impaired directional chemotactic accuracy, speed and velocity of migration towards recognised chemoattractants (fMLP and IL-8). The case-control study presented in this thesis represents novel and unique data in that a number of different neutrophil functions have been measured from the same patient and control volunteers. Neutrophil IL-8 release was greater from the patient compared to control neutrophils (Chapter 4) and IL-8 is a widely recognised chemoattractant used within the directional chemotaxis assay (section 2.2.10). It was therefore postulated that the amount of neutrophil IL-8 release by patients' neutrophils may negatively correlate with directional chemotactic accuracy and provide a causal mechanism for the observed defective neutrophil chemotaxis in chronic periodontitis (Chapter 6). If neutrophil IL-8 release was responsible for the defective chemotaxis observed, one would expect a negative correlation between neutrophil IL-8 and concurrent chemotactic index. However, in contrast, there was a moderately significant positive correlation between neutrophil IL-8 release and chemotactic accuracy towards fMLP (Chapter 6). Neutrophils are the predominant leucocyte of the innate immune system and, as such, will be recruited following initiation of an inflammatory-immune response and probably actively participate in the recruitment of distant inflammatory cells, including additional neutrophils. This is of *in vivo* relevance and important in disease pathogenesis given that greater neutrophil recruitment, albeit with impaired chemotactic accuracy, of a hyper-active and hyper-reactive neutrophil phenotype, will increase the probability of collateral host tissue damage. Reduced neutrophil chemotactic accuracy is unlikely to result in a significant delay in bacterial clearance from the gingival crevice given the large number of neutrophils present. However, greater tissue transit times would lead to increased release of potentially damaging neutrophil pro-inflammatory mediators, including cytokines, ROS and antimicrobial peptides (section 1.5.4), due to relatively erratic migratory pathways whilst travelling towards host and plaque-derived chemoattractants within the periodontal tissues.

Chronic periodontitis results in a systemic inflammatory-immune response with greater plasma C-reactive protein (CRP) concentrations compared to healthy controls (Paraskevas *et al.* 2008; Chapter 3). This was thought to be potentially due to hyper-reactive neutrophil IL-6 release (Chapter 4), since IL-6 is a primary trigger for CRP synthesis and release from liver hepatocytes (Gauldie *et al.* 1987, Gabay & Kushner 1999). However there was no correlation between unstimulated patient neutrophil IL-6 release and plasma CRP concentration ( $R^2=0.03$ ;  $P=0.5$ ; data not shown). Nonetheless, given the large numbers of neutrophils and their central role in the inflammatory-immune response, it remains possible that increased neutrophil IL-6 release may contribute in part to the systemic inflammatory response and increased systemic CRP concentrations observed in chronic periodontitis. However, currently the consensus from the 2012 European and American workshop on periodontal and systemic diseases is that periodontally-derived bacteraemia likely drives the acute-phase CRP response reported in periodontitis patients (Tonetti & Van Dyke 2013)

The effects of physiologically relevant concentrations of CRP, equivalent to those identified in chronic periodontitis (Chapter 3) and used as an indicator for cardiovascular disease risk (Ridker *et al.* 2002, 2005), on the neutrophil respiratory burst of control neutrophils from periodontally and systemically healthy volunteers were determined (Chapter 8). Unstimulated neutrophil HOCl and  $O_2^-$  generation and release, *F. nucleatum* stimulated HOCl and Fc $\gamma$ R-stimulated  $O_2^-$  release were all significantly inhibited in the presence of CRP. Raised systemic CRP concentrations may therefore be beneficial to the host in helping to reduce the oxidative stress associated with chronic periodontitis and systemic disease, and minimise the risk of co-morbidity. However, in contrast, increased *F. nucleatum* stimulated extracellular  $O_2^-$  release was observed in the presence of CRP. Whilst enhanced TLR-mediated  $O_2^-$  release from neutrophils may be beneficial in aiding host protection from infection in situations where acquired immunity has not yet been developed, this may also contribute to and exacerbate collateral host tissue damage in chronic periodontitis. Furthermore, greater *F. nucleatum* (TLR)-stimulated  $O_2^-$  in the presence of physiologically-relevant concentrations of CRP, even at the lowest 0.1 $\mu$ g/ml CRP concentration tested, may also represent a risk factor for systemic disease associated with oxidative stress in the presence of chronic periodontitis.

*Fusobacterium nucleatum* is a key periodontal pathogen, and periodontal bacteria and their products have the potential to enter the surrounding tissue and vasculature (Loos 2005, Seymour *et al.* 2007). As a result, it is likely that both *F. nucleatum* and increased concentrations of CRP are present in the peripheral blood of periodontitis patients. It is therefore interesting that there was a moderate, significant positive correlation between plasma CRP concentration of patient volunteers and their corresponding *ex vivo* neutrophil  $O_2^-$  release in the absence of overt stimulation (Figure 9.2). Thus it is possible that, in periodontitis, peripheral blood neutrophils in the presence of elevated CRP concentrations (Paraskevas *et al.* 2008; Chapter 3) and systemic circulating periodontal pathogens, including *F. nucleatum*, could be partly responsible for the observed neutrophil ROS hyper-activity following isolation from peripheral blood. This therefore highlights the importance of plaque control through oral hygiene and active periodontal treatment in reducing the potential for systemic *F. nucleatum*-CRP interaction resulting in the hyperactive and –reactive neutrophil phenotype in the pathogenesis of chronic periodontitis.



**Figure 9.2. Correlation (n=19) between unstimulated patient neutrophil  $O_2^-$  release and plasma hsCRP concentration (mg/l).** Superoxide detected by lucigenin in the absence of CRP (mean relative light units; RLU). Omission of the high outlying 20.8mg/l plasma hsCRP concentration results in a Pearson correlation of 0.591 ( $R^2 = 0.3493$ ;  $P < 0.01$ ).

Furthermore, in relation to the *in vivo* pathogenesis of chronic periodontitis, once neutrophil functions have been fulfilled in the tissues they undergo apoptosis to prevent the uncontrolled release of toxic cell contents (Fox *et al.* 2010). Apoptotic-specific cell changes promote the recognition and uptake of cells by phagocytes such as macrophages involving exposure of phosphorylcholine that becomes accessible to CRP only in apoptotic cells, and is therefore important in scavenging cellular debris and extracellular matrix components (Pepys & Baltz 1983, Szalai *et al.* 1999). It is therefore plausible that the greater systemic CRP concentrations in chronic periodontitis and systemic disease relate to the greater need for removal of apoptotic neutrophils during the inflammatory-immune response whilst, at the same time, also having both anti- and pro-inflammatory effects on viable neutrophil function. These include the aforementioned reduction in unstimulated and *F. nucleatum*-stimulated HOCl and FcγR-stimulated O<sub>2</sub><sup>-</sup> release, along with greater *F. nucleatum* stimulated O<sub>2</sub><sup>-</sup> release by neutrophils in the presence of CRP.

Overall, this thesis presents novel data that adds to our current understanding of neutrophil function and the host inflammatory-immune response in the pathogenesis of chronic periodontitis. Whilst the clinical relevance of this research may not be immediately apparent, it is hoped that greater understanding of the pathogenesis of chronic periodontitis will ultimately lead to improved preventative, diagnostic and treatment strategies in the future. Although oral hygiene, plaque control and elimination of periodontal risk factors (e.g. smoking), along with regular dental/periodontal examination, will remain the mainstay of prevention and therapy for periodontitis, it is possible that re-balancing the pro-inflammatory neutrophil phenotype through local or systemic adjunctive treatments may also be beneficial and improve periodontal treatment outcome in high risk patients. Moreover, greater understanding of neutrophil function in the presence of systemic inflammatory markers such as CRP increases the scientific evidence of the impact of systemic inflammation upon neutrophil function and risk of co-morbidity. This further adds to our current understanding of the associations between periodontal and systemic disease. It is therefore hoped that the risk of co-morbidity will be reduced in the future through greater understanding of the host response in chronic periodontitis, and ultimately improved periodontal risk assessment, diagnosis and treatment strategies in the future.

## 9.2 RECOMMENDATIONS FOR FUTURE RESEARCH

The data presented in this thesis fulfil the aim of contributing to our current understanding of the role of the neutrophil in the pathogenesis of chronic periodontitis. However a number of additional questions have not been addressed within this thesis, which will further contribute to our current understanding of the role of the host response in the pathogenesis of chronic periodontitis. Therefore recommendations for future research include:

- Performance of gene expression analyses on the stored neutrophil cell pellets following 18 hour culture in the presence and absence of periodontally relevant bacteria (section 2.2.6). This would provide novel information as to the transcriptional responses of neutrophils in response to periodontal bacteria to support the neutrophil cytokine release data (Chapter 4).
- A study of NET formation in response to periodontally-relevant bacteria from patients with chronic periodontitis and healthy controls following on from the PMA and HOCl-stimulated NET data presented in this thesis (Chapter 6). Whilst no significant differences in PMA and HOCl-stimulated NET release were detected in the current studies, this may be due to the NET stimuli used. For example, even the well-established neutrophil hyper-reactivity in chronic periodontitis in response to TLR and FcγR stimulation is not observed following PMA stimulation (Chapter 5). It is therefore possible that physiologically-relevant stimuli may result in differences in NET release not detected using PMA and HOCl. Moreover, the subsequent effect of non-surgical periodontal therapy could also be studied by performing an additional case controlled longitudinal intervention study.
- Assessment of the effect of non-surgical periodontal therapy on the impaired neutrophil directional chemotactic accuracy, speed and velocity of movement (Chapter 6). This data has already been collected and analysed as part of a collaborative project with H. Roberts (section 2.2).
- Determination of the effect of physiologically relevant concentrations of CRP on NET and cytokine release and directional chemotactic accuracy.



### **9.3 CONCLUSION**

This thesis has investigated a number of key neutrophil functions in chronic periodontitis, namely IL-8, IL-6, IL-1 $\beta$ , TNF- $\alpha$ , O<sub>2</sub><sup>-</sup> and NET release along with directional chemotactic accuracy. In addition, the effects of physiologically relevant concentrations of CRP on the neutrophil respiratory burst were investigated. Taken together, this thesis further supports current evidence demonstrating that a number of different neutrophil functions are dysregulated in chronic periodontitis. In addition, the effects of CRP on neutrophil respiratory burst activity further emphasise the impact of chronic periodontitis and systemic disease on the systemic inflammatory-immune response and subsequent risk of co-morbidity.

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# APPENDIX I

## Medical history questionnaire

Are you:	Yes	No	Details
1. Receiving medical or hospital treatment at present?			
2. Taking any tablets, medicines or any other substance e.g. inhalers?			
3. Allergic to any tablets, medicines or any other substance e.g. Penicillin/Latex (rubber)?			
4. Are you pregnant? (if appropriate)			
5. Have you ever had a heart murmur, rheumatic fever or any other problem with your heart?			
6. Have you ever had raised blood pressure, angina, heart attack or thrombosis e.g. CVA, DVT?			
7. Have you ever had hepatitis, jaundice or been diagnosed with HIV disease?			
8. Have you ever had any chest problems e.g. asthma/bronchitis or tuberculosis?			
9. Have you ever had an operation or illness treated in hospital?			
10. Have you ever been diagnosed with epilepsy?			
11. Have you ever been diagnosed with diabetes?			
12. Have you ever undergone brain surgery, received growth hormone treatment before the mid 1980's or do you have a close relative with CJD?			
13. Have you ever had prolonged bleeding following a tooth extraction or other surgery?			
14. Have you ever had a problem with LA or GA?			
15. Are there any other problems that may be relevant?			

# APPENDIX II

## Patient volunteer information leaflet

South Birmingham   
Primary Care Trust

Dental Services Directorate  
Birmingham Dental Hospital  
St. Chad's Queensway  
Birmingham  
B4 6NN

UNIVERSITY OF  
BIRMINGHAM  
SCHOOL OF DENTISTRY

### VOLUNTEER INFORMATION SHEET (GROUP 2)

Version 1.1 (2<sup>nd</sup> September 2010)

**Study Title:** Analysis of neutrophil response in chronic periodontitis study

**Sponsor Reference:** RG\_10-077

**NHS R&D Reference:** R&D1398

**REC Reference Number:** 10/H1208/48

<b>Researcher:</b>	Mr Martin Ling (PhD thesis)
<b>Contact Telephone No.</b>	
<b>Contact Email:</b>	
<b>Research Sponsor:</b>	University of Birmingham
<b>Sponsor Reference:</b>	RG_10-077
<b>Research Supervisor:</b>	Professor Iain Chapple
<b>Contact Telephone No.</b>	
<b>Contact Email:</b>	
<b>Research site:</b>	Birmingham Dental Hospital & School
<b>NHS R&amp;D Reference</b>	R&D1398
<b>Research Ethics Committee:</b>	West Midlands REC
<b>REC Reference Number:</b>	10/H1208/48

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

Please feel free to ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

**WHAT IS THE PURPOSE OF THE STUDY?**

Recent scientific evidence suggests that the defence cells of the body (neutrophils) behave differently in patients with gum disease, namely how they interact with bacteria and their role in defence systems of the body. There is also evidence that gum disease is related to other important diseases such as diabetes and rheumatoid arthritis. The aim of the study is to look at behavior patterns of these defence cells in relation to volunteers without gum disease. As a result we aim to learn more about the condition and hopefully develop new methods to treat or possibly even prevent the disease in the future.

**WHY HAVE I BEEN INVITED TO TAKE PART?**

You have been invited to take part in the study because you have good general health and require treatment for gum disease and your dentist has requested your treatment be performed at the Dental Hospital. This study will have two parallel groups, one test (gum disease) and two separate controls (no gum disease and one group with chronic granulomatous disease as a negative control).

**WHAT WILL HAPPEN TO ME IF I TAKE PART?**

If you decide to take part in this study you will be treated in exactly the same manner as all our patients who suffer from gum disease apart from some additional sampling procedures. These will include taking routine clinical measurements of your teeth and gums during two visits with the collection of gum fluid (which is painless to collect and takes 30 seconds) and a blood sample. We will also take two tissue samples from your gums on two of your four treatment visits in order to look at the cells under a microscope and from which some cells will be grown, stored until use and their behaviour tested against different challenges. These biopsies will be extremely small, approximately the size of a needle-head and will heal uneventfully within days with no detrimental effects to you or they way that your disease is managed whatsoever. There will be only one additional appointment (approx 20 minutes) for you to attend and treatment appointments will be slightly longer than normal (by approximately 30 minutes) so that the measurements and samples can be taken.

**DO I HAVE TO TAKE PART IN THE STUDY?**

It is up to you to decide whether or not to take part and is entirely voluntary. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**WHAT HAPPENS IF I WISH TO WITHDRAW FROM THE STUDY?**

Participation in the study is entirely voluntary and you may withdraw at any time. If you wish to withdraw from the study then you are free to do so without question and without giving a reason. To do this you will need to contact either Martin Ling (Researcher) on 0121 237 2806 or M.R.Ling@bham.ac.uk, or alternatively Professor Iain Chapple (Research Supervisor) on 0121 237 2808 or I.L.C.Chapple@bham.ac.uk. In the event of a volunteer wishing to withdraw from the research then all samples will be identified by the Researcher, destroyed and data deleted. Any samples already analysed would have been done so by study number only and as such will be non-identifiable to any other staff other than the Researcher. All data will be utilized in a non-identifiable manner.

**WILL MY TAKING PART IN THE RESEARCH BE CONFIDENTIAL?**

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. Your name will not be identified with the data collected other than by a code. Results collected will be expressed as an average for all volunteers participating in the research with no identifiable details whatsoever. Individual volunteer data will not be used in the publication of results.

## **WHAT WILL I HAVE TO DO?**

You are asked to read this Information Sheet, ask any questions you may have about the study and, if you wish to take part, sign the consent form. You will then be asked to fill out a separate medical questionnaire that will be used to help decide whether or not you meet the study requirements.

All information gathered about you will be treated in strict confidence. It may be made available to Regulatory Authorities, Auditors, the Ethics Review Group or the Study Monitors in order to check the quality of the data. If it is to be used for scientific purposes your name will not be disclosed.

There will be one extra appointment over standard treatment (approximately 20 minutes), and the first and review appointments will be slightly longer as will two of the four treatment visits. We expect the additional time taken will be approximately 1 hour (20 minutes per appointment). In addition the gum tissue samples will be taken during two out of your four treatment visits and we expect the additional time for these visits will again be approximately 1 hour (30 minutes per appointment). All study procedures will take place at the Dental Hospital.

### **VISIT 1: Identification and information presentation**

- You will have been identified as a potential patient volunteer for the study and you will have been given verbal and written information about participation in the study.

### **VISIT 2: Recruitment and GCF sampling**

**You must not drink anything, brush your teeth or chew gum within 2 hours as this may affect our results.**

- A separate appointment will then be arranged for you to ask any further questions and complete a medical history form, during which time we will assess whether you are suitable for the study.
- **If you are suitable for the study** we will answer any questions you may have and if you are willing to participate we will ask you to sign a consent form.
- If so we will complete some basic measurements of your gums, identically as if you weren't participating in the study as this forms part of your clinical care. This appointment will take approximately 20 minutes.
- We will collect gum fluid samples which will take approximately 5 minutes.

### **VISIT 3: Blood sampling**

- We will collect gum fluid samples which will take approximately 5 minutes
- We will take a blood sample (approximately ten teaspoons) which will take a couple of minutes.
- You will then proceed to have intensive oral hygiene instruction by a dedicated Staff Hygienist which will form your preliminary treatment visit, which would occur regardless of your participation in the study.

### **VISIT 4: First treatment visit and 1<sup>st</sup> set of gingival biopsy sampling**

- We will begin gum treatment in the first of four corners of the mouth, with treatment completed over four visits, which is exactly as it would be done if you were not enrolled in the study.
- We will use a local anaesthetic prior to treatment, exactly as we would if you were not enrolled in the study, and whilst anaesthetized we will collect two small gum tissue samples which will be approximately the size of a small needle-head immediately before commencing treatment, and whilst covered by the anaesthetic and will take approximately 20 minutes in

addition to your treatment. These are extremely small and will heal uneventfully within days with no detrimental effect on your treatment whatsoever.

- We will then continue your gum treatment exactly as we would were you not enrolled in the study.

#### **VISIT 5: Second treatment visit and 2<sup>nd</sup> set of gingival biopsy sampling**

- We will continue your gum treatment in the second of four corners of the mouth exactly as if you were not enrolled in the study.
- We will again use a local anaesthetic prior to treatment, and whilst anaesthetized we will collect an additional two small gum tissue samples in another corner of the mouth. These will again be approximately the size of a small needle-head and collected immediately before treatment whilst covered by the anaesthetic and will take approximately 20 minutes in addition to your treatment. These are extremely small and will heal uneventfully within days with no detrimental effect on your treatment whatsoever.
- We will then continue your gum treatment exactly as we would were you not enrolled in the study.

#### **VISIT 6: Third treatment visit (routine)**

- This will be an entirely routine gum treatment visit in the third of four corners of the mouth with the use of a local anaesthetic.

#### **VISIT 7: Fourth routine treatment visit (routine)**

- This will be an entirely routine gum treatment visit in the final corner of the mouth with the use of a local anaesthetic.

#### **VISIT 8: Three-month follow-up after treatment (routine) and additional blood/GCF sampling. You must not drink anything, brush your teeth or chew gum within 2 hours as this may affect our results.**

- We will take some routine measurements of your teeth and gums which will take approximately 20 minutes in order to assess your response to treatment as we would do were you not enrolled in the study.
- During this visit we will collect an additional sample of gum fluid which will take 5 minutes.
- We will also take an additional blood sample (approximately ten teaspoons) which will take a couple of minutes.
- We will then ask you to complete a feedback form so that we can gain your thoughts on participation in the study.

*At this point you will then exit the study and continue with your clinical care regardless of whether or not you have participated in the study.*

#### **WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?**

There are no disadvantages to taking part, except that 2 of your visits will be approximately 20 minutes longer and you will need blood and gum fluid samples taking at these visits. In addition, two of your four treatment visits will take approximately 30 minutes longer whilst we collect two small tissue samples from around your gums.

Venepuncture is a procedure where we obtain blood from the vein on the anterior forearm (the side within the fold of the elbow). This is a safe and commonly performed procedure although may result in minor discomfort and/or bruising which, if occurs, heals uneventfully. In addition there is a minor chance of infection at the site of blood collection, although this has never been experienced within the department in over twenty years. Blood will be taken by a suitably qualified member of clinical staff

and as such these risks are extremely minimal. All blood taken will be in accordance with the University of Birmingham “Blood Taking from Volunteers for Research” Health and Safety Policy document.

The tissue samples collected will be extremely small (2mm<sup>2</sup> – approximately the size of a needle-head) that will be taken from the gums surrounding two teeth during the first two out of four standard treatment visits. These will be the equivalent of creating a small ulcer at the site of treatment that will heal uneventfully within days with no detrimental effects whatsoever and will not affect the treatment received or its outcome in any way.

#### **ARE THERE ANY SIDE EFFECTS OF THE TREATMENT?**

The gum disease treatment you will receive is entirely that which you would routinely need. Although this in itself may make your gums shrink back a little, this happens with all gum treatments and is unavoidable if you want the disease treated. The additional tests you will require as part of the study (i.e. providing blood, gum fluid and gum tissue samples) have no side effects.

#### **WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?**

You will benefit by taking part because your treatment will be by an experienced member of staff rather than a student, which is the normal practice at the Dental Hospital. The treatment will therefore take less time than if you did not take part.

#### **WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?**

At the end of the study, you will enter a normal review process and any further treatment needed will be arranged with a member of staff or your dentist as is normal practice.

#### **WHAT IF SOMETHING GOES WRONG?**

We do not expect you to experience any problems as a result of taking part in this study. However, should you have any concerns then you should contact the study team on 0121 237-2792 or -2793 and ask to speak to someone involved in the Neutrophil Response (NR) study. If you have any general questions about the study please call Martin Ling on 0121 237-2806 or -2808 (office hours).

If you are harmed due to someone’s negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you. ‘The sponsor’ will not compensate you where such injury results from any procedure carried out which is not in accordance with the protocol for the study. Your right at law to claim compensation for injury where you can prove negligence is not affected.

#### **WILL I BE INFORMED OF THE RESULTS OF THE STUDY?**

Participants will not be individually informed of their results. However it is the aim to publish results of the research in peer-reviewed journals and as part of a PhD thesis, which will be freely available for access. However, if you wish to know results out of scientific interest then we will be happy to give you a copy of the final study report and also of any resulting publications.

#### **WHO IS ORGANIZING AND FUNDING THE RESEARCH?**

This study has been organized through The University of Birmingham as ‘Sponsor’, and has been planned within the Periodontal Research Group at the School of Dentistry. Funding has been secured through successful research grants awarded from the Oral and Dental Research Trust and the Royal College of Surgeons of England.



**WHO HAS REVIEWED THIS STUDY?**

All research in the NHS is looked at by an independent Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable ethical opinion by the West Midlands Research Ethics Committee.

**CONTACT FOR FURTHER INFORMATION?**

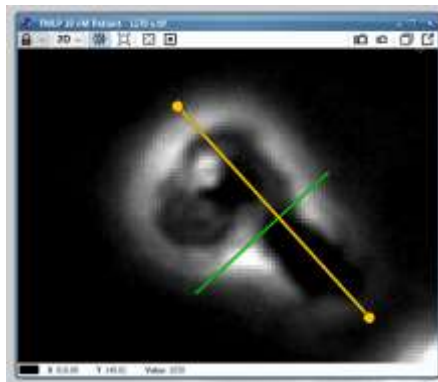
If you need further information please phone 0121 237-2792 or -2793 and ask to speak to someone involved in the “Neutrophil Response (NR)” study. This will be Martin Ling or one of the study team. Alternatively, general independent advice about taking part in research can be obtained from our PALS spokesman Derek de Faye on 0121 237 2836.

**Thank you for taking the time to read this information sheet**

## APPENDIX III

### Neutrophil elliptical factor analysis

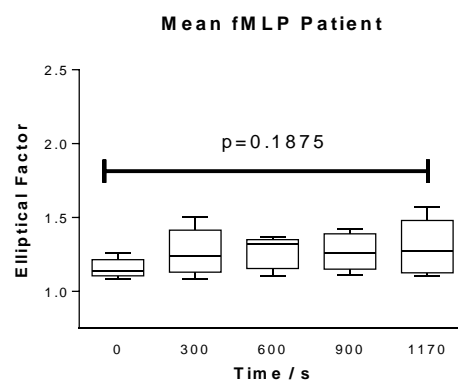
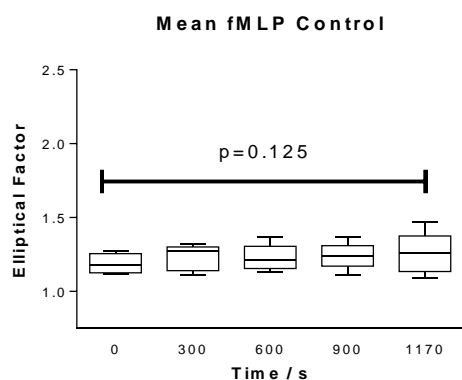
Neutrophils identified previously by Martin Ling & Helen Roberts were analysed to calculate their elliptical factor. Following consultation with Dr. Joshua Rappoport five images were used for the elliptical factor analysis (t=0 s, 300 s, 600 s, 900 s, 1170 s). The elliptical factors were calculated by measuring the greatest distance between the cell membranes (or observable edges) and the perpendicular distance (at 90 ° and midpoint), as shown below:



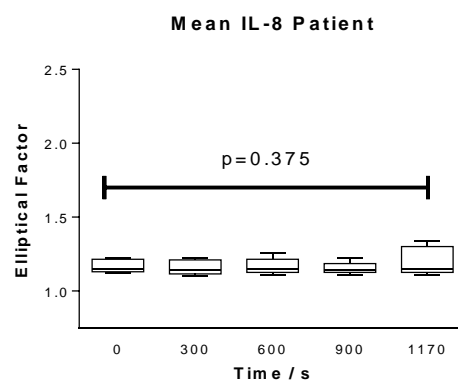
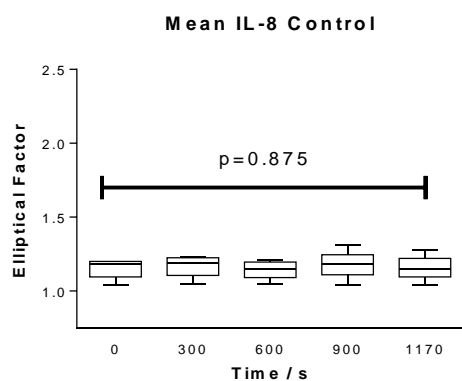
Up to 15 cells were analysed for each dataset (*i.e.* patient or paired control), depending on the ability to successfully identify the elliptical factor throughout the time course (*e.g.* if any cell moved partially off screen this cell would not be analysed).

A total of five paired patients and controls were analysed, and the mean elliptical factor of each patient and control sample was calculated. These mean values (n=5) were analysed with the use of Friedman and Wilcoxon tests (shown overleaf). As it can be seen, no statistical significance is identified between t=0 s and 1170 s (Wilcoxon test) or between patient and control samples.

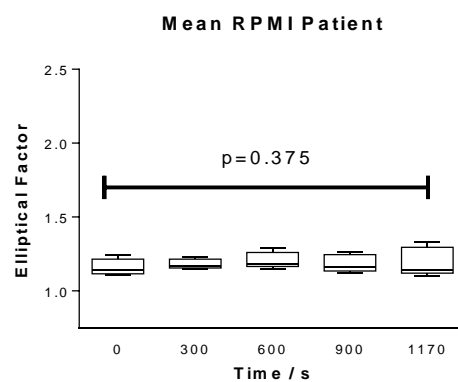
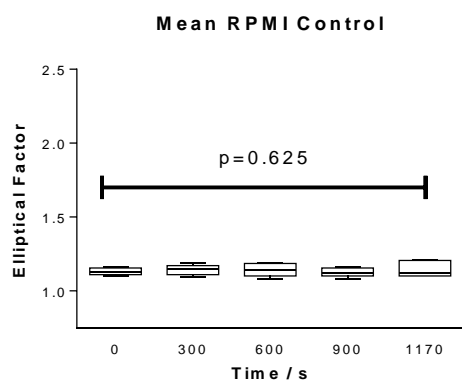
The Wilcoxon test shows that for fMLP the control cells have a greater elliptical factor at t=1170 s in comparison to the patient sample ( $P=0.125$  in comparison to  $P=0.1875$ ), whereas for IL-8 and RPMI the values are much lower for the patient. It is difficult to explain this finding, but it did appear that the cells were much more mobile (for both control and patient) when stimulated with fMLP than IL-8. As such it is possible that due to the increased chemotaxis (especially for the control sample) and high cell density the cells were increasingly interacting and moving around other cells, which would explain why they would be more elongated. With respect to IL-8 and RPMI, no statistical significance is identified, although in both cases the p value is approximately half of the control sample, which may suggest that if a greater  $n$  were analysed then significance may be seen.



Friedman test: No significance



Friedman test: No significance



Friedman test: No significance

# APPENDIX IV

## Research conference presentations

**Ling MR.** Chemotactic Accuracy of Peripheral Blood Neutrophils in Chronic Periodontitis using a Novel Direct Visualisation Chemotaxis Chamber. University of Birmingham College of Medical & Dental Sciences Festival of Graduate Research, 8<sup>th</sup> April 2014.

**Ling MR.** Cytokine release from peripheral blood neutrophils in chronic periodontitis. Periodontal Research Group, 13<sup>th</sup> March 2014.

**Ling MR.** Chemotactic Accuracy of Peripheral Blood Neutrophils in Chronic Periodontitis using a Novel Direct Visualisation Chemotaxis Chamber. Birmingham Inflammation Group, 7<sup>th</sup> March 2014.

**Ling MR.** Chemotactic Accuracy of Peripheral Blood Neutrophils in Chronic Periodontitis using a Novel Direct Visualisation Chemotaxis Chamber. University of Birmingham School of Dentistry Science Lunchtime Seminar, 21<sup>st</sup> February 2014.

**Ling MR, Roberts HM, Matthews JB, Grant MM, Chapple IL.** Chemotactic Accuracy of Peripheral Blood Neutrophils in Chronic Periodontitis using a Novel Direct Visualisation Chemotaxis Chamber. British Society of Periodontology Sir Wilfred Fish Research Presentation, The Royal College of Surgeons of England, London, October 2013.

**Ling MR, Chapple IL, Matthews JB.** Effects of C-Reactive Protein on the Neutrophil Respiratory Burst *In Vitro*. College of Medical & Dental Sciences, University of Birmingham, March 2013.

**Ling MR, Matthews JB, Chapple IL.** An Investigation into the Chemotactic Accuracy of Peripheral Blood Neutrophils from Patients with Chronic Periodontitis. The Alpha Omega London Chapter and Charitable Trust, London, UK, November 2012.

**Ling MR, Chapple IL, Matthews JB.** C-Reactive Protein Modulation of Peripheral Blood Neutrophil Response. Birmingham Inflammation Group, University of Birmingham, March 2012.

**Ling MR, Matthews JB, Chapple IL.** Enhanced Chemiluminescence Assays to Measure Neutrophil Reactive Oxygen Species. West Midlands Primary Immunodeficiency Group, University of Birmingham, November 2011.

**Ling MR, Chapple IL, Matthews JB.** Effect of C-Reactive Protein on Neutrophil ROS Production. The Royal College of Surgeons of England, London, UK, November 2011.

**Ling MR, Chapple IL, Matthews JB.** Effect of C-Reactive Protein on Neutrophil ROS Production. British Society for Oral and Dental Research, Sheffield, UK, September 2011.

**Ling MR, Chapple IL, Matthews JB.** C-Reactive Protein Modulation of Peripheral Blood Neutrophil Response (ID 207). International Association for Dental Research, San Diego, California, USA, March 2011.

# APPENDIX V

## Research papers published in peer-reviewed journals

**Roberts HM, Ling MR, Insall R, Kalna G, Grant MM, Chapple IL (2014).** Neutrophil chemotaxis in chronic periodontitis. Submitted to *J Clin Periodontol*

**Ling MR, Chapple IL, Creese AJ, Matthews JB (2014).** Effects of C-reactive protein on the neutrophil respiratory burst *in vitro*. *Innate Immun*; 20(4): 339-349

**Matthews JB, Chen FM, Milward MR, Ling MR, Chapple IL (2012).** Neutrophil superoxide production in the presence of cigarette smoke extract, nicotine and cotinine. *J Clin Periodontol*; 39(7): 626-634

**Palmer LJ, Cooper PR, Ling MR, Wright HJ, Huissoon A, Chapple IL (2012).** Hypochlorous acid regulates neutrophil extracellular trap release in humans. *Clin Exp Immunol*; 167(2): 261-268

**Grant MM, Creese AJ, Barr G, Ling MR, Scott AE, Matthews JB, Griffiths HR, Cooper HJ, Chapple IL (2010).** Proteomic analysis of a noninvasive human model of acute inflammation and its resolution: the twenty-one day gingivitis model. *J Proteome Res*; 9(9): 4732-4744

# APPENDIX VI

## Research grants

### **Birmingham & the Black Country Comprehensive Local Research Network funding allocation**

£15,938.00

May 2012

### **The Royal College of Surgeons of England Faculty of Dental Surgery Research Grant**

£9,200.00

November 2010

### **Oral & Dental Research Trust Glaxo Smith Kline Research Grant**

£6,273.00

August 2010

### **British Society of Periodontology Research Grant**

£4,444.00

April 2010

# APPENDIX VII

## Research prizes & awards

### **Sir Wilfred Fish Research Prize**

British Society of Periodontology  
October 2013

### **Highly Commended Award for doctoral research poster presentation**

The University of Birmingham College of Medical & Dental Sciences Festival of Graduate Research  
March 2013

### **Inaugural Dr John Zamet Memorial Prize in Periodontal Research**

The Alpha Omega London Chapter & Charitable Trust  
November 2012